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Features of Selective Kinase Inhibitors

Review

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Summary

Small-molecule inhibitors of protein and lipid kinases have emerged as indispensable tools for studying signal transduction. Despite the widespread use of these reagents, there is little consensus about the biochemical criteria that define their potency and selectivity in cells. We discuss some of the features that determine the cellular activity of kinase inhibitors and propose a framework for interpreting inhibitor selectivity.

Introduction

The dramatic clinical success of Imatinib has fueled an explosion in kinase-inhibitor discovery research [1]. It is estimated that kinase inhibitors currently comprise up to 30% of drug-discovery programs in the pharmaceutical industry, and over 50 such compounds are now in clinical trials [2]. The scale of this investment has led to the discovery of compounds with properties that scarcely resemble their early predecessors—picomolar potency, isoform selectivity, and allosteric binding modes are increasingly common [3–9]. As these reagents filter into the hands of scientists engaged in basic research, they will transform the study of signal transduction.

The first kinase inhibitors were described nearly 20 years ago [2], and a small subset of these compounds have found widespread application, forming the basis for much of what we know about the physiological roles of their targets. As we anticipate a new era of molecularly targeted agents, it is fair to ask what practical lessons have been learned from the use of these early compounds. What determines the potency of an inhibitor in cells? What is required for a kinase inhibitor to be selective, and how can this be measured? How is it possible to validate pharmacological results? The aim of this review is to suggest a framework for evaluating and using kinase inhibitors with a focus on the use of these reagents to explore signal transduction in cell-culture-based model systems.

The Relationship between Potency In Vitro and In Vivo

The potency of a kinase inhibitor for its target is typically expressed as an IC_{50} value—the concentration of drug at which 50% of the kinase activity is inhibited. Most kinase inhibitors are reversible and ATP competitive, and for these reagents, the IC_{50} depends on the intrinsic affinity of the inhibitor (the dissociation constant, K_i) as well as the competition from ATP under the specific assay conditions (the [ATP] and the $K_{M,\ ATP}$). These variables are related to each other by the Cheng-Prusoff equation [10]:

$$IC_{50} = K_i(1 + [ATP] / K_{M,\ ATP})$$

This equation captures the fact that at low ATP concentrations, there is no significant competition from substrate and the $IC_{50} \cong K_i$. As the ATP concentration exceeds the $K_{M,\ ATP}$, the IC_{50} increases at approximately the same rate (Figure 1A). Importantly, the IC_{50} does not plateau at a maximum value at high concentrations of ATP (in contrast to how an enzyme approaches V_{max} as the [ATP] exceeds the $K_{M,\ ATP}$).

For this reason, the potency of an inhibitor in cells (where the ATP concentration is 1–5 mM [11, 12]) depends critically on the $K_{M,\ ATP}$ of its target (Figure 1A). An inhibitor that has similar K_i values against multiple kinases will inhibit more potently in cells those kinases that have a higher $K_{M,\ ATP}$. We have assembled 238 published $K_{M,\ ATP}$ values for 111 protein and lipid kinases (Table 1 and Figure S1 available with this article online). The majority of these values are in the low- to midmicromolar range, and for these targets, ATP-competitive inhibitors should be active in cells at concentrations ~10- to 100-fold above their K_i. There are outliers, however, and these kinases will be more or less difficult to target with ATP-competitive small molecules. For example, several phosphatidylinositol 4-kinases and the related protein mTOR have millimolar $K_{M,\ ATP}$ values [13–17], and we have argued that trends in IC_{50} values for LY294002 analogs against phosphatidylinositol 3-kinases (PI3-Ks) can be explained largely by differences in affinity for ATP [13].

An important caveat to the use of $K_{M,\ ATP}$ values is that they are sensitive to the specific assay conditions (such as choice of protein substrate [18] or counter ion [19]). $K_{M,\ ATP}$ values for a single kinase measured under different conditions generally show small variation (<5-fold) (Figure S1) and, therefore, likely approximate the true in vivo substrate affinity of these enzymes. However, for some kinases $K_{M,\ ATP}$ values are lower when measured with protein substrates versus peptides [18] or when manganese is used in place of magnesium [20], and these factors must also be considered.

Most kinases are believed to interconvert between at least two structural conformations, active and inactive, and the phosphorylation of key residues can shift the balance between these states (Figure 1B). These two states are characterized by movements in conformationally mobile loops that border or block the ATP binding site (for example, the DFG motif). For this reason, the $K_{M,\ ATP}$ may be significantly higher for the inactive conformation than for the active conformation (Figure

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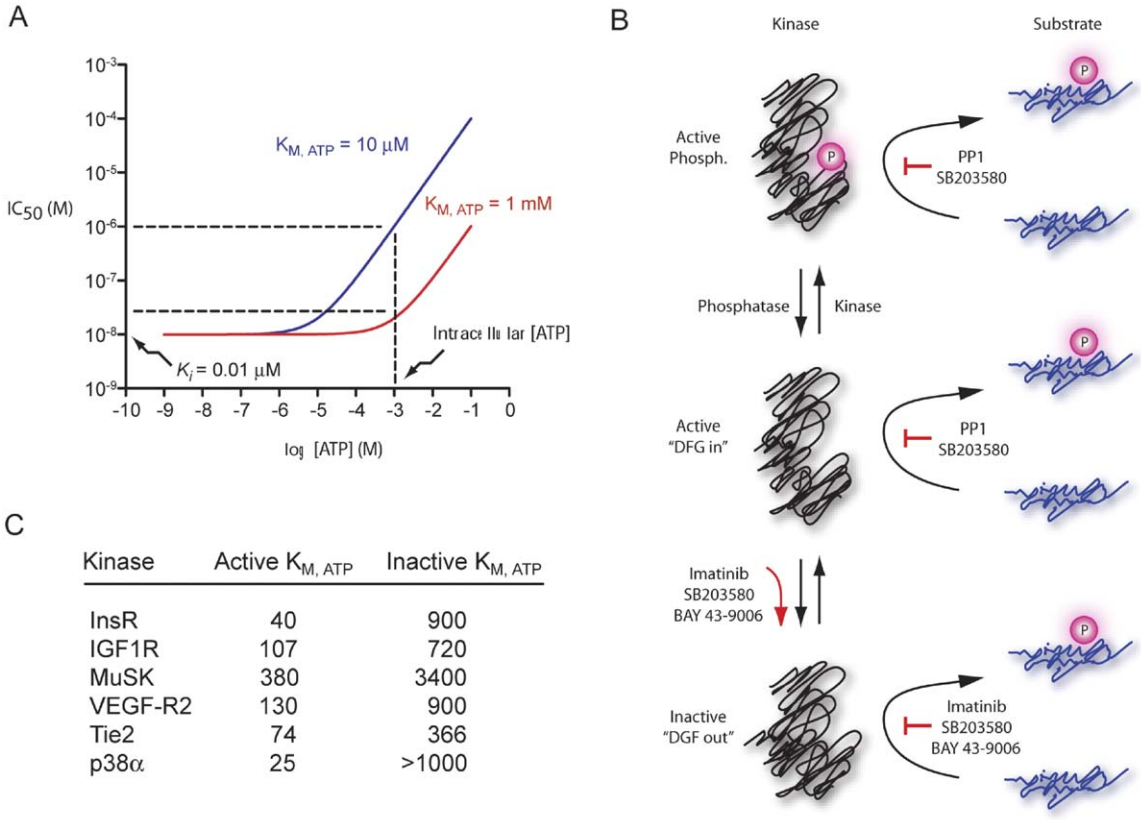

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Kinase	Active $K_{M, ATP}$	Inactive $K_{M, ATP}$
InsR	40	900
IGF1R	107	720
MuSK	380	3400
VEGF-R2	130	900
Tie2	74	366
p38α	25	>1000

Figure 1. The Cellular Potency of ATP-Competitive Kinase Inhibitors Depends on the $K_{M, ATP}$

(A) Theoretical relationship between $K_{M, ATP}$ and cellular potency for an ATP-competitive inhibitor with a K_i of 10 nM.

(B) A simplified schematic depicting how different classes of kinase inhibitors may block kinase activity.

(C) Examples of kinases that show large differences in $K_{M, ATP}$ between two phosphorylated forms.

1C). A growing number of kinase inhibitors selectively target the inactive conformation [3, 21, 22], whereas other compounds bind to both conformations with similar affinity [23]. Inhibitors that bind to the inactive conformation will face weaker competition from cellular ATP, and this may enhance their activity in vivo. Indeed, even though these compounds are ATP competitive, they may act primarily by shifting equilibria between conformational states in a way that prevents kinase activation, rather than by inhibiting kinase activity directly (Figure 1B). For example, the p38α inhibitor SB203580, which binds to both conformations, has been proposed to act in cells by stabilizing an inactive conformation that reduces the rate of p38α phosphorylation by MAPKKs [23].

Biochemical Activity Predicts Cellular Activity

Biochemical affinities are measured in vitro in order to predict concentration ranges at which kinase inhibitors will be active in cells. To what extent does kinase inhibition in cells actually correlate with in vitro measurements? To address this question, we analyzed published data for 13 classes of inhibitors that target three different protein kinases: VEGF-R2, IKK-2, and Lck. Figure 2 plots the relationship between biochemical K_i and EC_{50} for these compounds. EC_{50} is defined as the concentration of compound required to inhibit 50% of a cellular phenotype (different colors represent different structural classes of inhibitors, whereas different shapes represent different cellular assays). K_i values were estimated from in vitro IC_{50} values based on the reported assay conditions, and compounds were excluded that lacked potency in vitro (K_i > 1 μM) or had no activity at the highest concentration tested in cells (fewer than ten compounds). These values were then compared with predictions based on the consensus $K_{M, ATP}$ reported for these kinases assuming an intracellular ATP concentration of 2 mM (dotted lines).

Five chemotypes of VEGF-R2 inhibitors were analyzed. There was a correlation between biochemical affinity and cellular potency for these molecules that extended across structural classes and among analogs within a series. The EC_{50} for VEGF-R2 inhibitors was, on average, 10-fold above the K_i, and most compounds fell within a 5-fold window of this value (4- to 20-fold). To what extent do these values match in vitro predictions? The $K_{M, ATP}$ of the VEGF-R2 kinase domain has been measured for the phosphorylated (active) and unphosphorylated (inactive) states by at least two laboratories, and the reported values are similar (130/900 μM and 150/600 μM [24, 25]). It is not known whether these compounds target the active or inactive state, so we plotted the predicted relationship between EC_{50} and K_i

Table 1. Selected $K_{M, ATP}$ Values for Protein and Lipid Kinases

Tyr Kinases		Ser/Thr Kinases		Lipid/PIK Kinases	
c-Abl	12	Akt1	132	ATM (Mn)	29
v-Abl	18	Akt2	254	DNA-PK	228
Btk	29	Aurora 2	34	mTOR	1000
Csk	15	Cak1-*Sc*	5	p110α/p85α	62
EGFR	17	CaMKI	110	p110γ	7.5
EphA7	30	CaMKIIα	19	PI3KC2α	32
EphB3 (Mn)	2.7	CaMKIV	27	PI3KC2β	120
ErbB-2	27	CaMKKα	33	PI4KIIα	28
ErbB-4	37	CaMKKβ	33	PI4KIIIα	300
FAK (Mn)	4.3	CDK1/cyclin B	2.3	PI4KIIIβ	1000
FER	7.1	CDK2/cyclin A	23	PI(4)P(5)KI	25
c-Fgr	20	CDK2/cyclin E	3.6	PI(4)P(5)Kiα	27
FGFR	70	CDK4/cyclin D1	418	PI(4)P(5)Kiβ	33
Fyn	70	CDK4/cyclin D2	200	PI(4)P(5)Kiγ	39
IGFR	107	CDK5/p25	3.2	PI(5)P(4)KII	5
InsR	40	Chk1	1.4		
ITK	36	Chk2	3.3		
JAK1	15	CK1α	19		
JAK3	6	CK1β	12		
c-Kit	53.6	CK2α	13.9		
Lck	10	CK2β	8.8		
Lyn	35	CLK1 (Mn)	80		
MER	40	CTR1-*Ar* (Mn)	9.1		
MuSK	380	DAPK31	2.4		
PDGFRβ	15	DMPK	2.3		
c-Src	80	Erk2	140		
v-Src-cat	12	GRK1	2		
Syk	10	GRK2	60.8		
c-Tak	3.3	GRK3	88.8		
Tie-2	73.9	GRK5	23.8		
TrkA (Mn)	9	GRK6	111		
VEGF-R2	130	GSK3β	50.2		
c-Yes (Mn)	16	IKK-1/IKK-2	13		
Zap-70	3	IKK-2/IKK-2	18		
		IKK-i (Mn)	3.1		
		IRAK4	600		
		JNK2	39		
		JNK3α1	1.9		
		MAPKAPK2	43		
		MEK1	5.6		
		NIMA-*As*	69		
		p38α	25		
		p38γ	27		
		p90Rsk-B	35		
		PAK2	71		
		PhK	200		
		PKA-α	25		
		PKA-γ	9.1		
		PKC-α	24.3		
		PKC-βI	37.2		
		PKC-βII	20		
		PKC-ε	14.5		
		PKC-θ	49		
		PKG	5.1		
		PLK1	2.6		
		Raf-1	11.6		
		ROCK-I	4.5		
		ROCK-II	4.5		
		Sky1p-*Sc*	235		
		smMLCK	71		
		skMLCK	101		
		TBK-1 (Mn)	5.9		

When multiple values were available for a single kinase, a consensus value was selected, with preference given to values measured with magnesium, a protein substrate, and the active conformation of the kinase. A complete list, with phosphoacceptor data and references, is available online as Figure S1. (Mn) indicates value was measured in the presence of manganese. Sc (*Saccharomyces cerevisiae*), Xe (*Xenopus laevis*), Ar (*Arabidopsis thaliana*), Pb (*Schizosaccharomyces pombe*), Ca (*Candida albicans*), and As (*Aspergillus nidulans*).

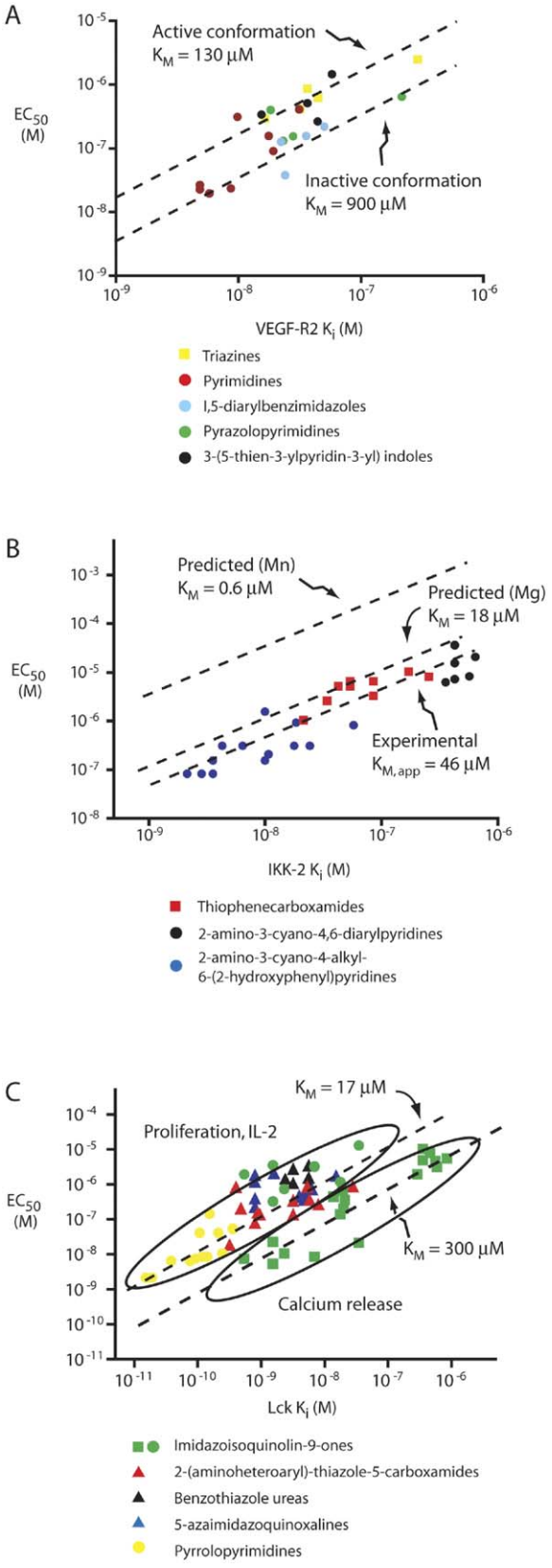


Figure 2. Correlation between K_i and EC_{50} for a Sample of Inhibitors of Three Protein Kinases

(A) VEGFR2: circles, VEGF-induced mitogenesis in HUVECs [119–122]; squares, VEGF-induced p42/p44 MAPK phosphorylation in HUVECs.

(B) IKK-2: circles, RANTES induction by TNFα in A549 cells [123–125]; squares, production of TNFα by HUVECs stimulated with LPS [125].

(C) Lck: circles, antibody-stimulated IL-2 secretion [126, 127]; squares, antibody-stimulated calcium release [127]; triangles, antibody-stimulated T cell proliferation [128–130].

for both conformations (dotted lines, Figure 2A). These two lines bracket most of the experimental values, suggesting that biochemical measurements predict the cellular activity of VEGF-R2 inhibitors with good accuracy.

We next analyzed data for inhibitors of IKK-2 (Figure 2B). IκB kinases exhibit $K_{M,\ ATP}$ values in the submicromolar range [26–30]—some of the lowest values reported for any protein kinase—suggesting that it may be necessary to use IKK inhibitors at high concentrations to achieve cellular activity (>1000-fold above K_i). Three classes of IKK-2 inhibitors were compared, and these compounds exhibit a strong correlation between biochemical K_i and EC_{50} across three orders of magnitude in inhibitor affinity (Figure 2B). Surprisingly the EC_{50} for IKK-2 inhibitors is, on average, only 44-fold above the K_i, corresponding to an effective $K_{M,\ ATP}$ of 46 μM for this kinase. What accounts for this discrepancy? One explanation is that the reported nanomolar $K_{M,\ ATP}$ values for IKK-2 (for example, 0.1, 0.14, 0.56, 0.6, and 0.65 μM [26–30]) were all measured under conditions that utilize manganese as a counterion. In the presence of only magnesium, the physiological divalent cation, the $K_{M,\ ATP}$ is 18 μM [31]—a value more consistent with the observed cellular activity of IKK-2 inhibitors. This highlights the fact that biochemical affinities measured with manganese are likely to be nonphysiological and may distort calculations of inhibitor potency.

Figure 2C depicts data for five chemotypes of Lck inhibitors, assayed according to their ability to block calcium release, IL-2 secretion, or proliferation of T cells. The cellular activity of these compounds falls into two classes. Calcium release is highly sensitive to Lck inhibition (mean $EC_{50} \cong 11\ K_i$, corresponding to an apparent $K_{M,\ ATP} \cong 300$ μM), whereas IL-2 production and T cell proliferation are much less sensitive, although almost identical to each other (mean $EC_{50} \cong 440\ K_i$, corresponding to apparent $K_{M,\ ATP} \cong 17$ μM). The latter value is close to the reported $K_{M,\ ATP}$ for Lck of 10 μM [32], suggesting that the potencies against IL-2 production and T cell proliferation are consistent with in vitro measurements, whereas calcium release is unexpectedly sensitive. This may reflect different thresholds for Lck activity for these two sets of processes and is consistent with the underlying differences in their kinetics—calcium release occurs in seconds, whereas cytokine production and proliferation occur over days. In general, these Lck inhibitors are also more varied in their cellular activity than IKK-2 or VEGF-R2 inhibitors. An important component of this variation is likely to be differences in the off-target activity of these inhibitors against other Src family kinases, such as Fyn, that are known to contribute to T cell signaling.

Sources of Deviation from Biochemical Predictions

The data from these three classes of inhibitors suggests that to a first approximation, biochemical affinities predict the cellular activity of kinase inhibitors remarkably well. Still, it is clear that many kinase inhibitors are more or less potent in cells than predicted by K_i and $K_{M,\ ATP}$, and it is certainly not possible to use these values to calculate an exact EC_{50} value. For example, the widely used PI3-K inhibitor LY294002 is consistently ~10-fold more potent in cells than biochemical measurements would predict. What mechanisms can account for discrepancies between inhibitor potency in vitro and in cells?

Phosphatases Are Endogenous Kinase Inhibitors

Phosphatases reverse the action of kinases in vivo, and this has the effect of systematically lowering the IC_{50} values for kinase inhibitors. This is because the net flux of phosphorylated product in the cell is the difference between the kinase and phosphatase activities. If the kinase and phosphatase turn over their substrates at similar rates, then inhibiting a small fraction of the kinase activity can block the entire flux of phosphorylated product (Figure 3A). A prediction of this model is that phosphatase inhibitors should decrease the potency of kinase inhibitors, and, indeed, inhibitors of the lipid phosphatase PTEN increase the cellular IC_{50} for LY294002 by ~5-fold [33]. Moreover, there is evidence that some signaling pathways are controlled by high levels of basal phosphatase activity. For example, treatment of lymphocytes with tyrosine phosphatase inhibitors can induce much higher phosphotyrosine levels than any physiological stimulus [34], suggesting that in these cells, the basal phosphatase activity is of the same magnitude as the stimulated kinase activity. For many signaling pathways, our understanding of the key phosphatases, their direct substrates, and their modes of regulation is limited compared to our understanding of the corresponding kinases. Yet every phospho-regulated step in signal transduction reflects a dynamic equilibrium between these two activities, and the relative phosphatase activity in each step will influence its sensitivity to small-molecule inhibition.

Kinase Reactions In Vivo Are Not Linear

IC_{50} values are measured in vitro in the presence of a large excess of phosphoacceptor substrate, such that the substrate concentration does not change significantly during the course of the assay (that is, only initial rates are measured). Under these conditions, the relationship between the concentration of active kinase (the fraction of the total kinase not inhibited by drug) and the concentration of phosphorylated substrate is linear (Figure 3B, black dashed line). This is not necessarily the case in the cell, where a kinase may phosphorylate a large fraction of its protein substrate; the fact that it is often possible to monitor protein phosphorylation by observing a molecular weight shift via SDS-PAGE is evidence that phosphorylation can occur at high stoichiometry in cells. Under these conditions, where phosphoacceptor substrate is significantly consumed, the concentration of active kinase and its phosphorylated substrate are related by a hyperbolic curve indicative of Michaelis-Menten kinetics (Figure 3B, red). In other cases, Ferrell and coworkers have shown that kinase-mediated signaling pathways, such as the MAP kinase cascade, can behave cooperatively, such that the concentration of active kinase and its phosphorylated substrate are related by a sigmoidal rather than hyperbolic curve [35, 36] (so-called ultrasensitivity) (Figure 3B, blue). This ultrasensitivity has been attributed to a number of features, including multisite phosphorylation of a single substrate, the activity of a kinase at multiple steps within a pathway, or the presence of several kinases arranged in a linear cascade.

The precise nature of the relationship between kinase activity and output for any given signaling pathway has implications for the sensitivity of kinases to small-molecule inhibition. Under Michaelis-Menten conditions, the biochemical IC_{50} (the concentration of drug at which half the kinase active sites are occupied by inhibitor in cells) is always lower than the EC_{50} (the concentration of drug at which half the total kinase activity is inhibited in cells) (Figure 3C). The magnitude of this effect can be large at very high stoichiometries of substrate consumption and tends to make kinase inhibitors appear less potent in cell culture than would be predicted by in vitro measurements. As the fraction of phosphoacceptor substrate that is consumed decreases (because of a weaker stimulus, a shorter time point, or a more abundant substrate), the hyperbolic Michaelis-Menten curve approaches in the limit the linear conditions observed in vitro, in which substrate is essentially unlimited and the biochemical IC_{50} and EC_{50} are identical.

Ultrasensitive behavior can make a kinase inhibitor seem more or less potent in cells, depending on the fraction of substrate that is consumed under the given assay conditions. At low substrate consumption, more than 50% of the net kinase activity can be inhibited by occupying less than 50% of the kinase active sites with inhibitor (Figure 3C). At high substrate consumption, the ultrasensitive response converges to the behavior of normal Michaelis-Menten kinetics.

Huang et al. have experimentally confirmed that the relationship between MAPKKK activity and the activity of its downstream effectors (MAPKK and MAPK) in *Xenopus* oocyte extracts exhibits ultrasensitivity, and this can be described by Hill coefficients of approximately two and five, respectively [36]. A consequence of this fact is that a MAPKKK inhibitor that blocks 50% of MAPKK activation will inhibit less than 50% of MAPK activation, even though these enzymes are directly connected in a linear signaling cascade (Figure 3D). Although most signaling pathways have not been characterized at this level of detail, many common mechanisms of signal transduction contain features that may introduce cooperativity and thereby perturb inhibitor sensitivity. For example, PI3-kinase activates the downstream kinase Akt by recruiting it and its upstream kinase PDK1 to the plasma membrane (Figure 3D). The quantitative relationship between PI3-K activity (PIP_3 generation) and Akt phosphorylation by PDK1 is unknown, but the simplest model is that Akt phosphorylation is a bimolecular reaction between Akt and PDK1, and, therefore, the rate is dependent on the concentration of each at the membrane ($v[pAkt] = k_1[Akt][PDK1]$). If the membrane concentration of each protein is proportional is to the concentration of PIP_3 ([Akt], [PDK1] $\propto$ [PIP_3]), then the rate of Akt phosphorylation should increase according to the square of the PIP_3 concentra-

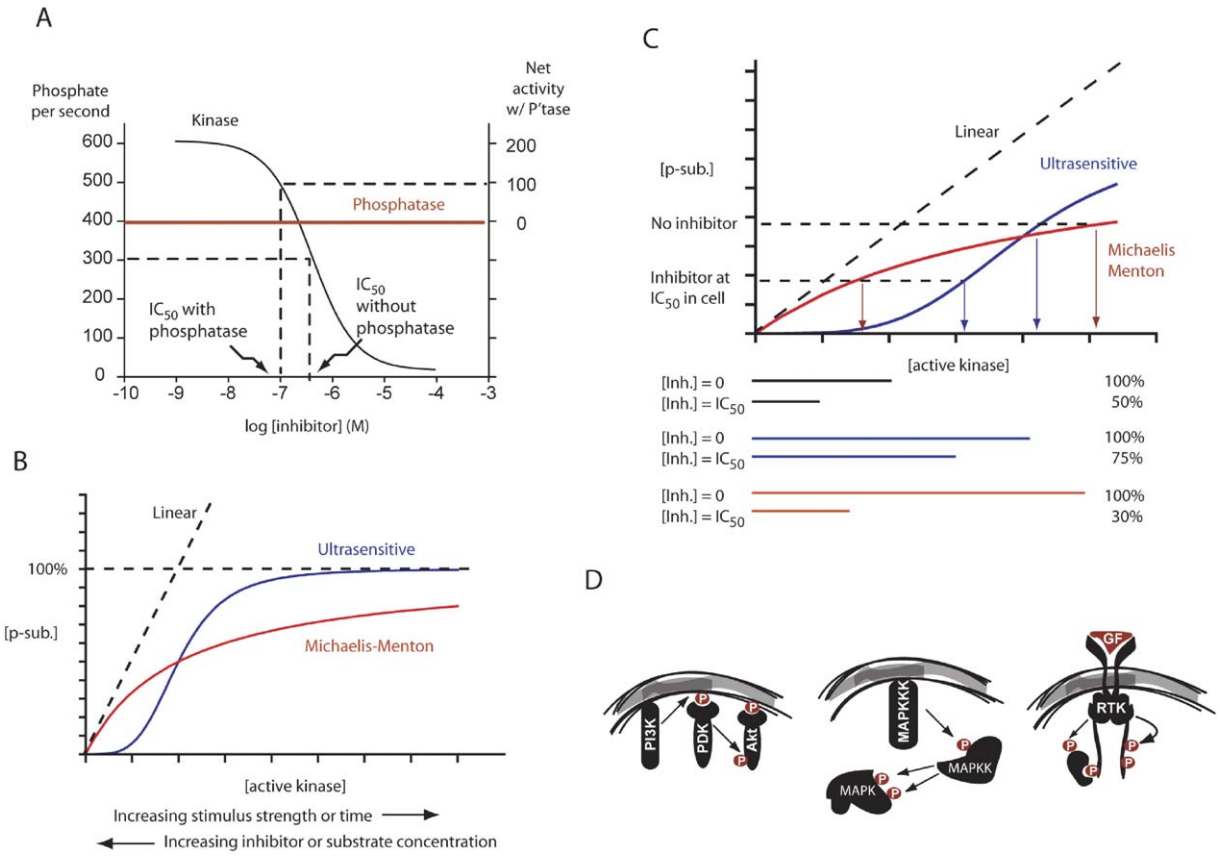


Figure 3. Factors that Can Influence Inhibitor Sensitivity in Cells

(A) Phosphatase activity can lower IC_{50} values for kinase inhibitors. In the absence of phosphatase activity, the IC_{50} for an inhibitor is the concentration at which half the kinase activity is inhibited (here, 600/2 = 300/s). In the presence of a fixed phosphatase activity of 400/s (red line), the net flux of kinase activity is reduced to 200/s. Therefore, inhibiting only 100/600 ≅ 16% of the direct kinase activity will block 100/200 ≅ 50% of the net flux of kinase activity. This model may be realistic for early time points, when the phosphatase activity is constant and independent of the kinase activity.

(B) The concentration of phosphorylated substrate ([p-substrate]) can have differential dependence on the concentration of active kinase ([active kinase]) depending on whether the activity obeys linear (dashed), Michaelis-Menton (red), or ultrasensitive (blue) kinetics [35]. Increasing the strength of the cellular stimulus or the length of time will shift the reaction to the right along these curves, whereas increasing the concentration of inhibitor or the stoichiometry of substrate will shift the reaction to the left.

(C) Detailed analysis of the effect that Michaelis-Menton or ultrasensitive kinetics can have on kinase sensitivity to inhibitors. For a linear response, the concentration of drug necessary to reduce the [p-substrate] by 50% corresponds to a 50% reduction in [active kinase] (that is, 50% of the kinase active sites are bound to drug). For Michaelis-Menton and ultrasensitive responses, reducing the [active kinase] by more or less than 50% is necessary to reduce the [p-substrate] by 50%.

(D) Schematic diagram of signaling in MAP kinase, PI3-K, and receptor tyrosine kinase (RTK) pathways.

tion ($v[pAkt] = k_{obs}[PIP_3]^2$). Under conditions of low substrate consumption in which this model may be realistic, PI3-K inhibitors should block Akt phosphorylation more potently than they block PI3-K activity directly (for example, inhibition of 50% of PI3-K activity should reduce phosphorylated Akt by 75%). A similar model would apply to a receptor tyrosine kinase, whose kinase activity both recruits its substrates via SH2 domain-phosphotyrosine interactions and subsequently activates them by direct phosphorylation (Figure 3D). It will be interesting to test these models experimentally in an effort to delineate the quantitative relationship between kinase inhibition and signaling output for different pathways.

Bioavailability of Kinase Inhibitors

Kinase inhibitors must enter the cell in order to inhibit their targets. The bioavailability of a kinase inhibitor in cell culture depends primarily on two factors. (1) How fast does the intracellular inhibitor concentration reach a steady state? (2) At the steady state, what are the relative concentrations of inhibitor inside and outside the cell? These questions depend largely on the magnitude of two rates: the rate at which the compound enters the cell by diffusion down the concentration gradient that exists across the membrane (the cell permeability) and the rate at which the compound is actively pumped out of the cell by efflux pumps.

For a given concentration gradient, the cell permeability of a compound is proportional to its lipophilicity (more lipophilic compounds will partition more readily from water into a membrane) and inversely proportional to its size. For this reason, molecules that are highly charged, have too many hydrogen bond donors and acceptors, or are very large cross cellular membranes

slowly. However, molecules that are extremely hydrophobic also have poor effective permeability either because they lack aqueous solubility, fail to partition out of the plasma membrane, or bind tightly to serum proteins. "Drug-like" kinase inhibitors tend to lack these structural defects, and these molecules often have very high rates of cell permeation. For example, levels of PIP_3 in insulin-stimulated 3T3-L1 cells sharply decline within ~1 min of LY294002 addition [37]. Actin rearrangements can be observed in T cells within 3–5 min of addition of the Lck inhibitor PP2 at a low dose (20 nM) [38]. Because it is customary to preincubate kinase inhibitors for 30–90 min prior to stimulation of the cell, it is safe to assume that most drug-like kinase inhibitors approach their steady state within the experimental time scale.

Inhibitors are also pumped out of the cell by drug efflux pumps. The rate of efflux depends on the intracellular inhibitor concentration, the K_M of a specific inhibitor for a given pump, and the overall level of pump activity. In some cases, the activity of efflux pumps can significantly lower the steady-state concentration of a drug. *S. cerevisiae*, for example, is resistant to many small-molecule inhibitors that are active in mammalian cells. Deletion of specific drug pumps renders yeast sensitive to most of these same compounds [39]. This is also the case for some tumor cells that overexpress transporters such as p-glycoprotein; expression of these transporters has been shown to reduce the steady-state intracellular drug concentration by up to 50-fold [40]. By contrast, most mammalian cells in culture appear to have less efflux activity.

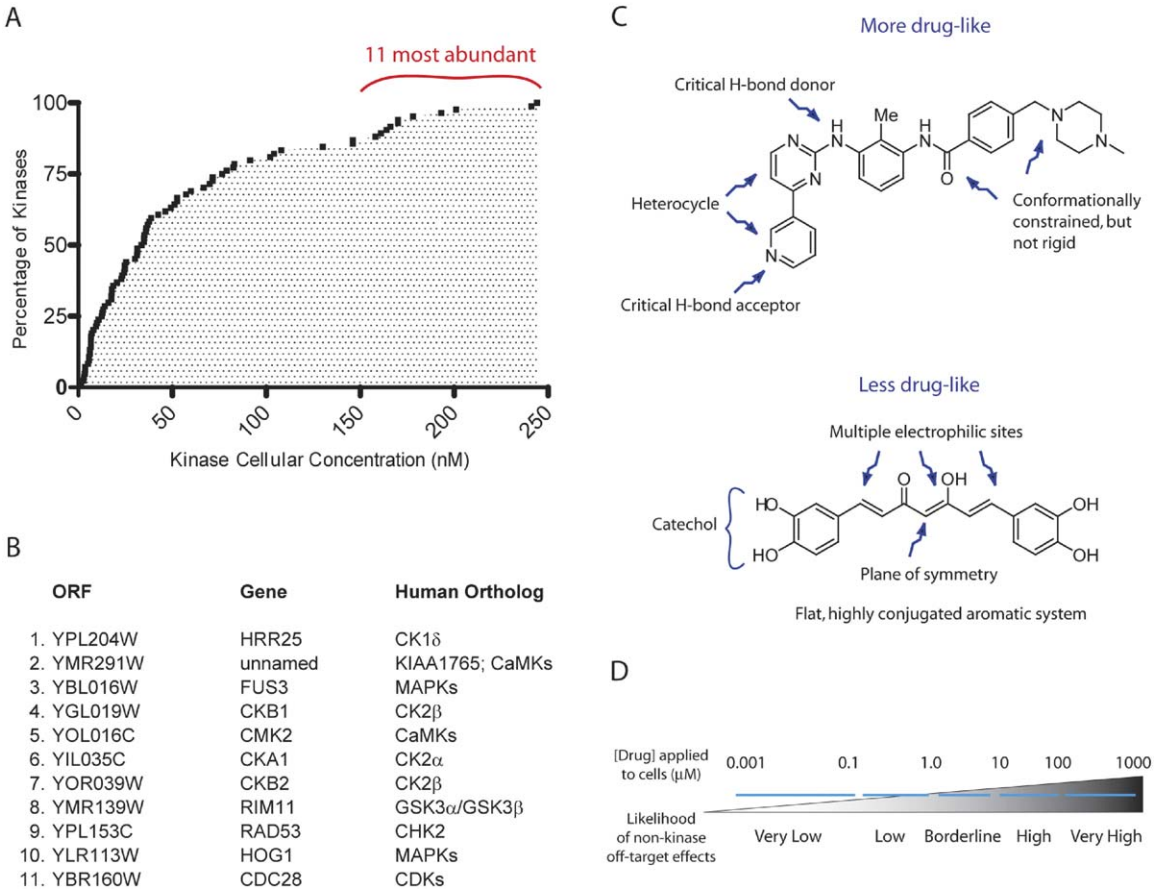
There is little published data that directly compares the bioavailability of different classes of kinase inhibitors in cell culture (bioavailability in animals is beyond the scope of this review). For drug-like kinase inhibitors with characteristically high rates of cell permeation, it seems likely that differences in steady-state intracellular drug concentration are the major source of experimental variation because of bioavailability. If this were not the case, then the potency of kinase inhibitors in cells would be highly sensitive to the length of preincubation; but this is not generally observed. An upper limit to the differences in steady-state bioavailability can be estimated indirectly by analyzing the scatter in plots such as Figure 2. Part of the deviation of this data from a straight line reflects differences in bioavailability across compounds (other factors include experimental error in measuring the in vitro and cellular IC_{50} values as well as differences in how these assays are conducted across different laboratories). For most of the compounds analyzed here, the cellular activity deviates from the mean within a 10-fold window for any given phenotypic endpoint. This represents an upper bound on the typical differences in bioavailability for potent, drug-like kinase inhibitors. Given that there are other significant sources of uncontrolled error in this analysis, this suggests that differences in bioavailability for kinase inhibitors in cell culture may be smaller than generally believed.

Kinases Are Low-Abundance Proteins

The lowest IC_{50} value that can be measured in vitro is determined by the concentration of kinase used in the assay. An IC_{50} value cannot be lower than one-half the concentration of kinase, because it is not possible to inactivate more than one kinase per molecule of drug (assuming a normal reversible binding mechanism). By extending this reasoning, it is sometimes argued that the potency of an inhibitor in cells depends on the intracellular concentration of its kinase target. A very abundant kinase will titrate a small molecule inhibitor out of solution such that the concentration of the kinase places a lower limit on the cellular IC_{50} for the inhibitor.

In most experimental settings, this reasoning is incorrect: the potency of a kinase inhibitor in cells should be independent of the concentration of its target (here, the concentration of kinase is distinguished from the amount of kinase activity). This is because kinase inhibitors are typically supplied in a large reservoir that can exchange matter with the cell, and the presence of a high affinity receptor within the cell will increase the steady-state intracellular drug concentration. For example, for most experiments in tissue culture, kinase inhibitors are added to the media and enter the cell through passive diffusion. Standard conditions for the growth of tissue culture cells—10^6 cells growing in a 10 cm dish bathed in 10 ml of media—correspond to a volume of cell culture media 10,000-fold greater than the volume of cells. In this regime, no change in the concentration of inhibitor within the cell can significantly alter the concentration of inhibitor in the media. For a potent inhibitor of an abundant kinase (K_i << [kinase]), the binding of the inhibitor to the kinase will therefore increase the total intracellular concentration of inhibitor because the driving force for diffusion across the membrane is primarily the concentration gradient of unbound inhibitor.

Natural variation in the expression levels of kinases can affect inhibitor sensitivity indirectly by changing the level of total kinase activity and, thereby, the number of turnovers needed to consume substrate or overcome a phenotypic threshold. For example, cell lines that overexpress Bcr-Abl can be made 3-fold more sensitive to Imatinib by reduction of Bcr-Abl levels via RNAi [41]. Limited data is available on the absolute expression level of most proteins in mammalian cells, but the expression of 80% of the predicted genes in yeast has been measured with an epitope-tag library [42]. This data set includes absolute expression levels for 84 of the 116 protein kinases predicted by the yeast genome, and we converted these values to protein concentrations by assuming a volume of 70 μm^3 for haploid *S. cerevisiae* [43] (Figure 4A). By this calculation, 63% of protein kinases are expressed at a concentration between 1 and 50 nM, and only 11 kinases (13%) are expressed at higher than 150 nM, with a maximum concentration of ~240 nM. By comparison, the median protein concentration in *S. cerevisiae* is ~54 nM, and the most abundant protein is expressed at ~38 μM. These calculations do not include values for the 38% of kinases for which expression data is unavailable, although the absence of data likely reflects the extremely low abundance of many of these proteins. Available data from mammalian cells and *Xenopus* oocytes is generally consistent with these concentrations, although some highly abundant kinases such as MAPKs and CDKs can be expressed as high as 1–2 μM [35, 44, 45]. These values provide some insight into the abundance of specific kinases within the protein-kinase superfamily and relative to other cellular proteins; how-



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	ORF	Gene	Human Ortholog
1.	YPL204W	HRR25	CK1δ
2.	YMR291W	unnamed	KIAA1765; CaMKs
3.	YBL016W	FUS3	MAPKs
4.	YGL019W	CKB1	CK2β
5.	YOL016C	CMK2	CaMKs
6.	YIL035C	CKA1	CK2α
7.	YOR039W	CKB2	CK2β
8.	YMR139W	RIM11	GSK3α/GSK3β
9.	YPL153C	RAD53	CHK2
10.	YLR113W	HOG1	MAPKs
11.	YBR160W	CDC28	CDKs

Figure 4. Biochemical Properties of Kinases and Kinase Inhibitors

(A) The fraction of kinases that are expressed at less than a given cellular concentration in *S. cerevisiae*.

(B) The 11 most highly expressed protein kinases in *S. cerevisiae*.

(C) Comparison of drug-like properties of Imatinib and a curcumin derivative.

(D) Proposed general guidelines for estimating the likelihood of off-target (nonkinase) effects at different concentration ranges of inhibitor applied to cells.

ever, it remains necessary to empirically determine the relationship between expression levels and inhibitor sensitivity for any specific kinase.

The Distribution of Pharmacological Variation

We have highlighted some of the reasons why the potency of kinase inhibitors in cells may deviate from biochemical predictions. This is not to suggest it is possible to predict these deviations in any specific case. The important fact is that kinase inhibitors have been successfully used to dissect signaling pathways, and this implies that the sources of variation in inhibitor potency must be small in magnitude and poorly correlated, such that their net effect causes modest overall deviations from in vitro predictions. This result was not guaranteed. Indeed, the effectiveness of kinase inhibitors in cells is entirely contingent on the fact that evolution has tuned the biochemical activities of kinases to phosphorylate a significant fraction of their substrates, but not much more, during the time course of an ordinary stimulus. If kinases were endowed with significant excess catalytic capacity—for example, 100-fold more activity than needed to phosphorylate 90% of their available substrate—then it would be, for all practical purposes, impossible to make a kinase inhibitor.

The identification of different sources of pharmacological variation has implications for kinase-inhibitor selectivity, even if it is not possible to predict the magnitude of these effects. Highly related kinases (for example, isoforms within a family) are more likely to share many sources of variation—such as a common phosphatase, similar levels of specific activity relative to substrate abundance, and downstream effectors with similar kinetics and thresholds of activation. The presence of these shared signaling components reduces the number of potential sources of deviation for the in vivo activity of different small-molecule inhibitors of these targets. This argues, counterintuitively, that closely related kinases should be easier to selectively inhibit in cells, given a fixed level of biochemical selectivity of a small-molecule inhibitor.

Structural and Biochemical Features of Selective Inhibitors

Selective Inhibitors Are Drug-like Molecules

Most selective kinase inhibitors are drug-like molecules. Even though a kinase inhibitor can be a useful research tool without the functional properties of a drug

(for example, oral bioavailability), drug-like compounds strike an appropriate balance between aqueous solubility and cell permeability [46], both of which are necessary for activity in cells. Furthermore, nondrug-like molecules often have structural properties that compromise their selectivity. In the words of Lipinski and Hopkins, "chemical features associated with failure in drug discovery tend to cause compounds to have 'promiscuous' effects in biological systems" [47].

Several approaches have been advanced to define "drug likeness" by identifying the chemical features shared by orally active drugs. Lipinski's rule of five identifies upper limits on the molecular weight (<500 Da), hydrophobicity (ClogP $\leq$ 5), and the number of hydrogen bond donors ($\leq$ five) and acceptors ($\leq$ ten) that most drugs possess [48]. An alternative proposal by Veber has emphasized the fact that oral absorption is favored by low polar surface area (less than 140 Å^2) and decreased ligand flexibility (ten or fewer freely rotatable bonds) [49].

Beyond specific drug-like properties, selective kinase inhibitors are almost always heterocycles, rather than peptides, lipids, or other substrate analogs (for example, see Figure 4C). They are typically entropically constrained, with four or fewer freely rotatable bonds connecting any two ring systems. Most importantly, they exhibit dramatic structure-activity relationships (SARs). For example, almost all ATP-competitive kinase inhibitors satisfy at least one of the hydrogen bonds that is made between the adenine ring of ATP and the kinase [50]. Substitution of this hydrogen bonding atom can result in a ~1000-fold loss in affinity.

Many nonspecific kinase inhibitors share a distinct set of structural features (Figure 4C). Promiscuous compounds often have dye-like structures—flat, highly conjugated polyaromatic systems. These compounds tend to be hydrophobic, bind proteins nonspecifically, and aggregate at high concentrations [51]. Certain chemical moieties, such as catechols, are commonly found in low-specificity inhibitors such as many flavones and tyrphostins. Catechols have been shown to undergo cellular oxidation to generate more reactive species that probably account for some of the in vivo activity of these molecules [52, 53]. This oxidation can also be catalyzed in vitro by manganese, a common component of many kinase assay buffers, and this has been shown to contribute to the biochemical potency of catechols from four different scaffold classes [54]. Another feature common to low specificity kinase inhibitors is a plane or axis of symmetry. Although symmetric molecules are prevalent in screening libraries, kinase active sites are asymmetric. For this reason, symmetry is usually an indication that a molecule has not undergone target-directed chemical optimization.

Shoichet and coworkers have defined a mechanism for nonspecific inhibition in vitro that involves the formation of submicrometer aggregates [51, 55]. This aggregate formation is time dependent, sensitive to protein concentration, and reversible by detergents and may form the basis for the in vitro activity of low-specificity kinase inhibitors such as quercetin and rottlerin [55]. However, it is important to emphasize that this type of aggregation has been observed only at micromolar concentrations. For an ATP-competitive kinase inhibitor, a micromolar K_i alone is compelling evidence that the compound is not selective.

Kinase inhibitors containing electrophiles, such as Michael acceptors or α-haloketones, generally exhibit poor stability and increased off-target effects. For example, the electrophilic natural product wortmannin has a half-life in tissue culture media of ~10 min [56], whereas vinyl nitriles, such as those found in U0126, can undergo rearrangements on storage in DMSO [57]. Although electrophiles are frequent hits from screening libraries, it is generally not possible to optimize these leads because their activity is based on more chemical reactivity rather than target-specific contacts [58]. Nonetheless, recent examples of selective electrophilic inhibitors have been reported [59] (M. Cohen et al., submitted). An essential feature of these compounds is that they possess a core scaffold that binds reversibly to the kinase with moderately high affinity ($K_i < 1$ μM). This scaffold then positions a relatively deactivated electrophile in proximity to a nucleophilic residue found in a subset of targets in the kinase superfamily. Absent this tight binding, reversible core structure, it is not possible to attain a high degree of selectivity with an irreversible inhibitor.

Inhibitor Selectivity Is Measured In Vitro

The target selectivity of a kinase inhibitor is typically measured by profiling its activity against a panel of kinases in vitro. Kinases most closely related in primary sequence are most likely to share inhibitor sensitivity [60], and these are the most important targets to test. In some cases, impressive selectivity against a panel of closely related kinases can suggest a high degree of selectivity against the remainder of the kinome. For example, BAY 61-3606 inhibits Syk with a K_i of 7.5 nM and exhibits $\geq$1000-fold selectivity against the related tyrosine kinases Src, Fyn, Lck, Btk, and Itk, suggestive of a high degree of selectivity against less similar kinases [61]. In most cases, it is important to sample a broad range of kinases from different kinase subfamilies. Two valuable studies have profiled many of the most commonly used kinase inhibitors against 28 protein kinases in this way [62, 63].

The limitation of this approach is that even the largest kinase panels test only ~20% of the kinome and, therefore, may miss important targets (although for smaller families, such as the lipid kinases, near-exhaustive coverage is feasible [13]). Despite this limitation, specific features beyond sequence homology have been identified that predict kinase inhibition, and these can be used to select the best targets for testing. For example, the size of a single amino acid in the ATP binding pocket—termed the gatekeeper residue—has been shown to be a critical determinant of inhibitor sensitivity [64, 65]. Kinases with a threonine at this position are sensitive to a range of inhibitors, whereas those with a larger residue are broadly resistant. Approximately 50% of tyrosine kinases contain a threonine gatekeeper, compared to 10% of serine-threonine kinases. For this reason, serine-threonine kinases that possess a threonine gatekeeper (for example, Raf and p38α) are often sensitive to tyrosine kinase inhibitors, and vice versa [64, 66]. In the same way, certain pairs of kinases are known to exhibit similar pharmacological profiles (so-called SAR homology [67]). For example, GSK3β inhibitors often inhibit CDKs and TGFβ-R inhibi-

tors frequently inhibit p38α, even though these pairs of kinases possess limited sequence homology. Likewise, there is growing appreciation that PI3-K inhibitors tend to inhibit the related protein kinase DNA-PK much more frequently than other PI3-K-related kinases (PIKKs) such as ATM and ATR [13, 68].

Affinity chromatography offers a way of identifying inhibitor targets that is complementary to in vitro measurements. In this approach, the inhibitor is linked to solid support and used to enrich for cellular binding proteins, which are then identified by mass spectrometry. This approach has been successfully used to identify unexpected cellular targets of kinase inhibitors such as SB203580, which was shown to inhibit RICK more potently than its known target, p38α [66]. Improvements in methodology have made this an increasingly viable strategy for target identification; immobilized pyrido[2,3-d]pyrimidines have recently been used to enrich for over 20 kinases from cell lysates [69]. A limitation of affinity chromatography, however, is that it is biased toward more abundant proteins. Four kinase families—CDKs [70–74], CK1 isoforms [68, 70], MAP kinases [66, 70–72, 75], and GSK3β [66, 71, 74, 76]—account for a disproportionate fraction of the kinase targets that have been identified by affinity-based approaches. This reflects, in part, the relative cellular abundance of these proteins, each of which is an ortholog of one of the most highly expressed kinases in yeast (Figure 4B). Consistent with this view, subsequent biochemical analysis often reveals that the bait compound inhibits these kinases weakly in solution [66, 70, 72, 74, 75]. For this reason, affinity chromatography may identify new targets of an inhibitor but does not validate inhibitor specificity.

Selectivity Depends on Potency

There are on the order of 20,000 unique protein receptors in the cell, and it is impossible to test any significant fraction of these targets. At high compound concentrations, it becomes increasingly likely that an inhibitor will bind to these off-target sites. For this reason, the practical selectivity of a compound depends on its potency—more potent compounds are more selective because they can be used at a lower dose. This is also true within the protein-kinase family, in which there is a strong correlation between inhibitor potency and selectivity [67]. An important corollary of this fact is that there is a minimum threshold of potency without which a molecule *cannot* be selective, irrespective of any in vitro data.

The PI3-K inhibitors LY294002 and wortmannin illustrate this point. Both have been extensively profiled in vitro and show similar specificity profiles at their respective concentration ranges. These two compounds inhibit the class I PI3K's most potently, with mixed activity against the other PI3-K family members [13] and an off-target activity against two protein kinases: CK2 (LY294002), sMLCK (wortmannin), and PLK1 (both) [77]. Yet these compounds are not equally selective in cells because wortmannin is used at 1000-fold lower concentrations. Several new targets of LY294002 have recently been identified, including calcium channels, potassium channels, phosphodiesterases, and the estrogen receptor [78–81], and these are inhibited in the concentration range that is commonly used to inhibit PI3-Ks. A similar spectrum of targets has not been identified for wortmannin *at the low nanomolar concentrations* at which it inhibits PI3-Ks.

We have proposed guidelines for estimating the likelihood of off-target effects across different concentration ranges (Figure 4D). This estimation is based in part on the observation that the fraction of small molecules that bind to a protein in vitro is generally low at nanomolar concentrations but increases dramatically above ~10 μM. This is structure dependent—certain compound classes are more promiscuous than others [82, 83]. Most selective kinase inhibitors have low nanomolar K_i values and, so, are applied to cells at concentrations less than 10 μM, reducing the likelihood of off-target effects. Unfortunately, many of earliest and most commonly used kinase inhibitors are significantly less potent, and conclusions based on the use of these reagents are suspect. Compounds used at concentrations above 100 μM (for example, the PIKK inhibitor caffeine) are unlikely to have any significant selectivity.

A New Generation of Allosteric Kinase Inhibitors

Most kinase inhibitors are ATP competitive, which reflects the fact that ATP binding pocket presents a large hydrophobic surface that can bind small molecules with high affinity. It is much more difficult to find compounds that bind to other regions of protein kinases. However, in some cases, it has been possible to identify such molecules, and, once identified, they possess advantages over their ATP-competitive counterparts. Because these inhibitors do not compete with cellular ATP, they can typically be used at concentrations closer to their biochemical K_i. Also, residues outside the ATP binding pocket tend to be less conserved, opening the possibility for greater selectivity. In certain cases noncompetitive inhibitors can be substrate selective, inhibiting the activity of a kinase against only a subset of its targets.

The first noncompetitive kinase inhibitor to be discovered was rapamycin, a cyclic macrolide natural product that inhibits the protein kinase mTOR. Rapamycin acts by binding to the ubiquitously expressed protein FKBP [84], and it is this rapamycin-FKBP complex that binds to the FRB domain of mTOR [85, 86]. The FRB domain is N terminal to the mTOR kinase domain, and it is not understood how this binding event inhibits mTOR activity [87]. In cells, mTOR resides as part of two large (~2 MDa) protein complexes, termed mTORC1 and mTORC2 [88, 89]. Remarkably, only the first of these complexes is rapamycin sensitive. mTORC1 signals to increase translation in response to nutrients and growth factors, and this complex can be isolated by immobilized rapamycin-FKBP [88, 89]. mTORC2 signals to the actin cytoskeleton in response to the same stimuli, and this complex is rapamycin insensitive [88, 89]. Potent, ATP-competitive inhibitors of mTOR have not been reported, but such compounds will be an important tool for elucidating signaling through mTORC2.

The MEK1 inhibitor PD098059 was the first synthetic noncompetitive kinase inhibitor to be described [90]. This compound acts by binding to inactive MEK1 and preventing its phosphorylation by the upstream kinase Raf [91]. The key to the discovery of this compound was the use of a biochemical screen based on reconsti-

tution of the MAP kinase cascade in vitro; because this screen utilized a low activity form of MEK1, it was possible to identify a noncompetitive inhibitor of MEK1 activation [93]. Several subsequent allosteric inhibitors of MEK1 and MEK2 have been described, including U0126 and PD184352 [92, 93]. The recent crystal structural of a PD184352 analog in complex with MEK1 confirms that these compounds bind to a site adjacent to but not overlapping with the ATP binding pocket [94]. Moreover, the low degree of sequence conservation in this region of the kinase explains the high selectivity of these compounds [94].

For many years, the MEK inhibitors were an isolated example of potent, synthetic kinase inhibitors that bind to an allosteric site. Recently, however, several new allosteric inhibitors have been described. Scientists from Merck have reported several classes of compounds, including a series of pyrazinones (Figure 5), which are allosteric inhibitors of Akt. These compounds are noncompetitive with ATP, show selectivity between the isoforms Akt1 and Akt2, and bind to a region that includes the Akt-PH domain [4]. BMS-345541 has been reported as an allosteric inhibitor of IKK-2 that displays potent activity in an animal model of inflammation [5] (Figure 5). Several classes of nanomolar noncompetitive inhibitors of p38α have recently been described, along with extensive structural and biophysical characterization of their binding sites [3, 95]. Remarkably, at least one of these compounds is substrate selective, blocking p38α phosphorylation of MAPKAP2 but not ATF-2 [95].

The Intersection of Pharmacology and Genetics

Target Validation with Resistant and Analog-Sensitive Alleles

The classic way to confirm the phenotypically relevant target of a small molecule is to use a mutant allele of the kinase that has altered sensitivity to the inhibitor. For example, the TOR proteins were identified as the target of rapamycin through a screen for yeast mutants resistant to rapamycin [85]. Ecoptic expression of inhibitor-resistant allele of p38α has been used to confirm that p38α is the target of SB203580-mediated blockade of certain inflammatory responses [96]. Most recently, the identification of Bcr-Abl mutations that block Imatinib binding from CML patients refractory to Imatinib treatment confirms that Bcr-Abl is a clinically relevant target of this molecule [97].

A related approach is to use a kinase allele that is sensitive to a small molecule inhibitor that does not inhibit any wild-type kinase. For protein kinases, mutation of the gatekeeper residue to alanine or glycine can generate such analog-sensitive (as) kinase alleles [98]. By replacing the endogenous copy of the kinase with the as allele, the effects of inhibiting that kinase in a model system can be studied with a highly specific inhibitor. A key feature of this approach is that it is possible to directly confirm that the phenotype is due to inhibition of the as kinase by performing a control experiment in which cells expressing the wild-type kinase are treated with the same inhibitor.

Resistant and analog-sensitive alleles are complementary approaches to studying kinase function. The former asks whether inhibition of a kinase is necessary for a phenotype, whereas the latter asks if it is sufficient. Resistance mutations are typically used in the last stages of target validation, after an inhibitor, phenotype, and putative kinase target have been identified. By contrast, analog-sensitive alleles can be used in a discovery setting to identify new biological processes that are sensitive to inhibition of a specific kinase.

Knockouts and Inhibitors Can Yield Different Phenotypes

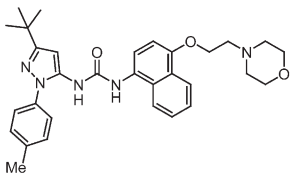
Genetic techniques such as RNAi and knockout animals offer an alternative to small-molecule inhibitors to study kinase function. RNAi in particular has great utility because it can be used to rapidly inactivate specific genes in cell culture. It is frequently proposed that RNAi might be used to validate targets for small molecule inhibition or confirm results from pharmacological experiments. Is this reasonable? Setting aside the fact that RNAi is itself a pharmacological intervention—with its own dose-dependent specificity limitations [99]—this belief reflects an underlying assumption that genetic knockdown of a kinase should phenocopy small-molecule inhibition [100].

There are many reasons to conclude this is incorrect [101]. Most kinases are multidomain proteins, and these other domains often possess kinase-independent functions [102, 103]. In some cases, the kinase domain itself has noncatalytic activity [104, 105]. It would be difficult to construct an accurate genetic model for an inhibitor such as rapamycin, which blocks a subset of mTOR's cellular functions by a complex mechanism, yet this compound was the first small-molecule kinase inhibitor approved for clinical use [106]. Most importantly, knockout mice for many kinases have surprisingly few detectable phenotypes [107]—indicating that other kinases may be able to mask the function of the knocked-out gene through compensation [108, 109].

Chemical inhibition of as-kinase alleles makes it possible to directly compare phenotypes of chemical and genetic kinase knockouts with inhibitors that have validated "single-target" specificity. These experiments indicate that small-molecule kinase inhibitors rarely, if ever, precisely phenocopy the corresponding gene knockout (Table 2). The knockout frequently elicits phenotypes not observed with the inhibitor [110, 111] (likely because of noncatalytic scaffolding functions of the kinase); the inhibitor induces phenotypes not observed in the knockout [98, 112] (likely because of compensation for the knockout by a homologous kinase); and, in at least one case, the inhibitor elicits the exact opposite phenotype as the knockout [104] (because of a noncatalytic, allosteric role of the kinase domain in signal propagation). These observations mirror studies comparing knockout mice and with mice expressing a kinase-dead allele (which better mimics the effects of a small molecule inhibitor). In many cases, the phenotypes are quite different [103, 113–115].

An additional layer of complexity arises from the fact that very few inhibitors target a single protein kinase, and the biological activity of these molecules may depend on a complex balance of inhibition of multiple targets. For example, CML, a disease characterized by the chromosomal translocation that generates the Bcr-Abl oncogene, may define the simplest link between genotype and kinase-inhibition phenotype. Yet it is

Compound	Primary Target	Selectivity	Cellular Activity/Binding Mode
BAY 61-3606	Syk: K_i = 7.5 nM	700 to >1000-fold for Src, Lyn, Fyn, Itk, and Btk	Blocks cytokine release from mast cells at low nanomolar concentrations
BMS-509744	Itk: K_i = 16 nM	50-100-fold for IR, Fyn, Lck, Btk; ≥ 1000-fold for 14 other kinases	Blocks IL-2, proliferation in T-cells at mid-nanomolar concentations
CP-690550	JAK3: K_i ~ 1 nM	20 and 100-fold for JAK2 and JAK1; >3000-fold for other 30 kinases	Blocks IL-2 induced T-cell proliferation at 11 nM
BIRB-796	p38α: K_i = 0.097 nM	>1000-fold against 13 other kinases	Blocks TNFα release in THP-1 cells at 18 nM; binds inactive conformation
BMS-243117	Lck: $K_i \cong 4$ nM	32, 60, and 84-fold for Fyn, Fgr, and Blk; >150-fold for other Src family kinases, >6000-fold for other PKs	Blocks T-cell proliferation at 1.1 μM
BMS-345541	IKK-2: $K_i \cong 300$ nM	10-fold for IKK-1; >300-fold for 15 other kinases	Allosteric, non-ATP competitive; blocks IκBα phosphorylation in cells at 4 μM
Pyrazinone 13b	Akt1: $K_i \cong 760$ nM	30-fold against Akt2; >100-fold against other AGC kinases	Allosteric, non-ATP competitive; pro-apoptotic in A2780 cells at 12 μM
Pyrazinone 14f	Akt2: $K_i \cong 325$ nM	65-fold against Akt1; >100-fold against other AGC kinases	Allosteric, non-ATP competitive; pro-apoptotic in A2780 cells at 12 μM
Naphthyridine 19	ALK5: $K_i \cong 4$ nM	>4000-fold against 9 other kinases including p38α	Blocks TGFβ induced reporter gene transcription at 18 nM
STI-571 (Imatinib, Gleevec)	Bcr-Abl: K_i = 14 nM	Inhibits PDGFR and c-Kit at similar concentrations; >1000-fold for many other kinases	Binds to the inactive conformation; active in cells at ~ 1 μM
ZD1839 (Gefitinib, Iressa)	EGFR: $K_i \cong 0.4$ nM	>50-fold selectivity against ErbB-2 and ErbB-4; >1000-fold for many other kinases	Similar in structure and selectivity to OSI-774 (Tarceva)
BAY 43-9006	B-Raf: K_i = 22 nM	Inhibits PDGFR, c-Kit, Flt-3, and VEGFR at similar concentrations; >1000-fold for many other kinases	Binds to the inactive conformation; active in cells at 0.1 to 1 μM
KU-55933	ATM: K_i = 2.2 nM	>100-fold selectivity against other PI3-Ks; >1000-fold selectivity against 60 protein kinases	Blocks p53 phosphorylation induced by ionizing radition at ~ 300 nM; radiosensitizes cells at ~ 10 μM
IC87114	p110δ : $K_i \cong 20$ nM	≥ 100-fold selectivity against all other PI3-Ks and many protein kinases	Active in cells at 0.1 to 5 μM



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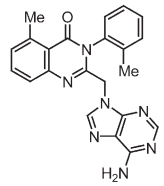

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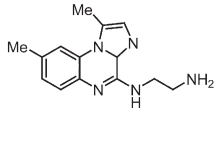

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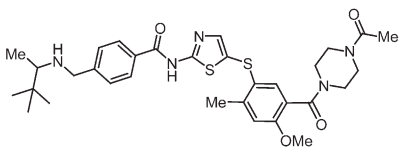

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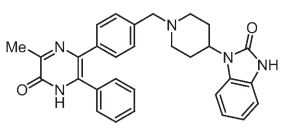
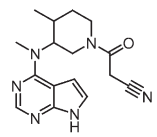


Pyrazinone 13b



CP-690550

Figure 5. Recent Examples of Selective Kinase Inhibitors

Table 2. Examples of Chemical and Genetic Kinase Knockouts that Produce Different Phenotypes

Kinase	Knockout Phenotype	Inhibition Phenotype	Proposed Explanation
CDC28	CDC28-ts allele arrests in G1 at restrictive temperature	Inhibition of the CDC28-as1 allele induces arrest at G2/M at low doses and G1 at high doses	The mitotic checkpoint is more sensitive to CDK activity than the G1 checkpoint
Ire1	Ire1Δ or Ire1-kd cells have defective unfolded-protein response (UPR)	Inhibition of an Ire1-as allele that also contains a kd mutation permits the UPR	An ATP competitive inhibitor of Ire1 permits activation of its RNAse domain during the UPR
Apg1	Apg1Δ cells are defective in cytoplasm to vacuole targeting (Cvt) as well as autophagy	Inhibition of Apg1-as allele or expression of Apg1-kd blocks Cvt but not autophagy	Cvt requires the catalytic activity of Apg1, whereas autophagy requires a scaffolding function
Cla4	Cla4Δ cells have defective septin localization to the bud neck	Inhibition of Cla4-as allele has no effect on septin localization	Septin localization may depend on a scaffolding function of Cla4
Elm1	Elm1Δ cells undergo G2/M delay	Inhibition of elm1-as allele results in G1 delay in bud emergence and Cln2 synthesis, as well as G2/M defect	Compensation for G1 defect in elm1Δ cells by accummulation of suppressors during culture
p110γ	P110$\gamma^{-/-}$ mice show increased cardiac contractility and tissue damage	p110γ-kd mice have normal cardiac function	p110γ interacts with PDE3B and regulates heart contractility independent of kinase activity

References: CDC28 [98], Ire1 [104], Apg1 [110], Cla4 [111], Elm1 [112], p110γ [103, 113].

clear that Imatinib's activity requires more than Bcr-Abl inhibition in some settings. The Imatinib sensitivity of murine myeloid leukemia cells that express both Bcr-Abl and c-Kit is dependent on Imatinib's ability to inhibit c-Kit [116]. Inhibition of Bcr-Abl is necessary but not sufficient to induce apoptosis in these cells. In this case, the Imatinib activity against c-Kit was an unintended by-product of the drug-discovery process, and this sort of multitargeted activity would be challenging to engineer into a compound based on predictive genetic models. Similarly, combined inhibition of Kin28 and Srb10 as alleles yields a synergistic inhibition of RNA-polymerase-II-mediated gene transcription that cannot be predicted by single-gene inactivation [117].

Why Bother?

It is not easy to use kinase inhibitors to dissect signaling pathways with high selectivity. A great deal of focused, target-driven chemistry is required to find a single potent compound. The scale of this task is such that the best compounds today are developed largely by the pharmaceutical industry. Once a potent compound is identified, its selectivity must be extensively characterized in vitro for it to have any real usefulness—and even then, it is impossible to test all of the potential targets. We have proposed guidelines for evaluating kinase inhibitor selectivity, but even in the best case, the possibility of confounding off-target effects cannot be eliminated.

Is there any good reason to use small-molecule kinase inhibitors rather than competing genetic approaches such as RNAi? We have emphasized that these two types of reagents perturb signaling pathways in different ways and, therefore, can give different outcomes. Small molecules can inhibit catalytic activity without affecting other protein domains that might be disrupted by a knockout. Small molecules are also fast acting and reversible and, thereby, can escape cellular compensation that might mask a relevant phenotype.

Perhaps the best reason to use kinase inhibitors to study signal transduction is so that we might understand the inhibitors themselves. The major barrier to developing new drugs is target validation [101, 118]—the challenge of predicting how inhibition of a target will translate into phenotype in a physiological setting. Different types of approaches can contribute to solving this problem, but pharmacology occupies a privileged position because it is the ultimate mode of intervention. No disease can be treated with a mutation (yet), and no genetic experiment can reliably predict the outcome of targeting a pathway with a small molecule. For this reason, it is critical to understand how potent and selective kinase inhibitors function in physiologically relevant model systems, even if the specific molecules themselves are not destined to be drugs. The emergence of a new generation of kinase inhibitors presents a unique opportunity to do this—by using these reagents to systematically redefine signaling pathways according to their pharmacological properties.

Supplemental Data

Supplemental Data include one figure and Supplemental References and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/6/621/DC1/.

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References

1. Druker, B.J. (2004). Molecularly targeted therapy: have the floodgates opened? Oncologist *9*, 357–360.
2. Cohen, P. (1999). The development and therapeutic potential of protein kinase inhibitors. Curr. Opin. Chem. Biol. *3*, 459–465.
3. Pargellis, C., Tong, L., Churchill, L., Cirillo, P.F., Gilmore, T.,

Graham, A.G., Grob, P.M., Hickey, E.R., Moss, N., Pav, S., et al. (2002). Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. Nat. Struct. Biol. *9*, 268–272.

4. Barnett, S.F., Defeo-Jones, D., Fu, S., Hancock, P.J., Haskell, K.M., Jones, R.E., Kahana, J.A., Kral, A.M., Leander, K., Lee, L.L., et al. (2004). Identification and characterization of pleckstrin homology domain dependent and isozyme specific Akt inhibitors. Biochem. J. *385*, 399–408. Published online September 29, 2004. 10.1042/BJ20040849
5. Burke, J.R., Pattoli, M.A., Gregor, K.R., Brassil, P.J., MacMaster, J.F., McIntyre, K.W., Yang, X., Iotzova, V.S., Clarke, W., Strnad, J., et al. (2003). BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. J. Biol. Chem. *278*, 1450–1456.
6. Arnold, L.D., Calderwood, D.J., Dixon, R.W., Johnston, D.N., Kamens, J.S., Munschauer, R., Rafferty, P., and Ratnofsky, S.E. (2000). Pyrrolo[2,3-d]pyrimidines containing an extended 5-substituent as potent and selective inhibitors of lck I. Bioorg. Med. Chem. Lett. *10*, 2167–2170.
7. Sadhu, C., Dick, K., Tino, W.T., and Staunton, D.E. (2003). Selective role of PI3K delta in neutrophil inflammatory responses. Biochem. Biophys. Res. Commun. *308*, 764–769.
8. Changelian, P.S., Flanagan, M.E., Ball, D.J., Kent, C.R., Magnuson, K.S., Martin, W.H., Rizzuti, B.J., Sawyer, P.S., Perry, B.D., Brissette, W.H., et al. (2003). Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science *302*, 875–878.
9. Lin, T.A., McIntyre, K.W., Das, J., Liu, C., O'Day, K.D., Penhallow, B., Hung, C.Y., Whitney, G.S., Shuster, D.J., Yang, X., et al. (2004). Selective Itk inhibitors block T-cell activation and murine lung inflammation. Biochemistry *43*, 11056–11062.
10. Cheng, Y., and Prusoff, W.H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. *22*, 3099–3108.
11. Traut, T.W. (1994). Physiological concentrations of purines and pyrimidines. Mol. Cell. Biochem. *140*, 1–22.
12. Gribble, F.M., Loussouarn, G., Tucker, S.J., Zhao, C., Nichols, C.G., and Ashcroft, F.M. (2000). A novel method for measurement of submembrane ATP concentration. J. Biol. Chem. *275*, 30046–30049.
13. Knight, Z.A., Chiang, G.G., Alaimo, P.J., Kenski, D.M., Ho, C.B., Coan, K., Abraham, R.T., and Shokat, K.M. (2004). Isoform-specific phosphoinositide 3-kinase inhibitors from an arylmorpholine scaffold. Bioorg. Med. Chem. *12*, 4749–4759.
14. Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C., and Thomas, G. (2001). Mammalian TOR: a homeostatic ATP sensor. Science *294*, 1102–1105.
15. Downing, G.J., Kim, S., Nakanishi, S., Catt, K.J., and Balla, T. (1996). Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. Biochemistry *35*, 3587–3594.
16. Zhao, X.H., Bondeva, T., and Balla, T. (2000). Characterization of recombinant phosphatidylinositol 4-kinase beta reveals auto- and heterophosphorylation of the enzyme. J. Biol. Chem. *275*, 14642–14648.
17. Suer, S., Sickmann, A., Meyer, H.E., Herberg, F.W., and Heilmeyer, L.M., Jr. (2001). Human phosphatidylinositol 4-kinase isoform PI4K92. Expression of the recombinant enzyme and determination of multiple phosphorylation sites. Eur. J. Biochem. *268*, 2099–2106.
18. Hawkins, J., Zheng, S., Frantz, B., and LoGrasso, P. (2000). p38 map kinase substrate specificity differs greatly for protein and peptide substrates. Arch. Biochem. Biophys. *382*, 310–313.
19. Tian, G., Kane, L.S., Holmes, W.D., and Davis, S.T. (2002). Modulation of cyclin-dependent kinase 4 by binding of magnesium (II) and manganese (II). Biophys. Chem. *95*, 79–90.
20. Sondhi, D., Xu, W., Songyang, Z., Eck, M.J., and Cole, P.A. (1998). Peptide and protein phosphorylation by protein tyrosine kinase Csk: insights into specificity and mechanism. Biochemistry *37*, 165–172.
21. Schindler, T., Bornmann, W., Pellicena, P., Miller, W.T., Clarkson, B., and Kuriyan, J. (2000). Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science *289*, 1938–1942.
22. Wan, P.T., Garnett, M.J., Roe, S.M., Lee, S., Niculescu-Duvaz, D., Good, V.M., Jones, C.M., Marshall, C.J., Springer, C.J., Barford, D., et al. (2004). Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell *116*, 855–867.
23. Frantz, B., Klatt, T., Pang, M., Parsons, J., Rolando, A., Williams, H., Tocci, M.J., O'Keefe, S.J., and O'Neill, E.A. (1998). The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. Biochemistry *37*, 13846–13853.
24. Kendall, R.L., Rutledge, R.Z., Mao, X., Tebben, A.J., Hungate, R.W., and Thomas, K.A. (1999). Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J. Biol. Chem. *274*, 6453–6460.
25. Parast, C.V., Mroczkowski, B., Pinko, C., Misialek, S., Khambatta, G., and Appelt, K. (1998). Characterization and kinetic mechanism of catalytic domain of human vascular endothelial growth factor receptor-2 tyrosine kinase (VEGFR2 TK), a key enzyme in angiogenesis. Biochemistry *37*, 16788–16801.
26. Sadler, T.M., Achilleos, M., Ragunathan, S., Pitkin, A., LaRocque, J., Morin, J., Annable, R., Greenberger, L.M., Frost, P., and Zhang, Y. (2004). Development and comparison of two nonradioactive kinase assays for I kappa B kinase. Anal. Biochem. *326*, 106–113.
27. Li, J., Peet, G.W., Pullen, S.S., Schembri-King, J., Warren, T.C., Marcu, K.B., Kehry, M.R., Barton, R., and Jakes, S. (1998). Recombinant IkappaB kinases alpha and beta are direct kinases of Ikappa Balpha. J. Biol. Chem. *273*, 30736–30741.
28. Mercurio, F., Murray, B.W., Shevchenko, A., Bennett, B.L., Young, D.B., Li, J.W., Pascual, G., Motiwala, A., Zhu, H., Mann, M., et al. (1999). IkappaB kinase (IKK)-associated protein *1*, a common component of the heterogeneous IKK complex. Mol. Cell. Biol. *19*, 1526–1538.
29. Kishore, N., Huynh, Q.K., Mathialagan, S., Hall, T., Rouw, S., Creely, D., Lange, G., Caroll, J., Reitz, B., Donnelly, A., et al. (2002). IKK-i and TBK-1 are enzymatically distinct from the homologous enzyme IKK-2: comparative analysis of recombinant human IKK-i, TBK-1, and IKK-2. J. Biol. Chem. *277*, 13840–13847.
30. Huynh, Q.K., Boddupalli, H., Rouw, S.A., Koboldt, C.M., Hall, T., Sommers, C., Hauser, S.D., Pierce, J.L., Combs, R.G., Reitz, B.A., et al. (2000). Characterization of the recombinant IKK1/IKK2 heterodimer. Mechanisms regulating kinase activity. J. Biol. Chem. *275*, 25883–25891.
31. Wisniewski, D., LoGrasso, P., Calaycay, J., and Marcy, A. (1999). Assay for IkappaB kinases using an in vivo biotinylated IkappaB protein substrate. Anal. Biochem. *274*, 220–228.
32. Flint, N.A., Amrein, K.E., Jascur, T., and Burn, P. (1994). Purification and characterization of an activated form of the protein tyrosine kinase Lck from an *Escherichia coli* expression system. J. Cell. Biochem. *55*, 389–397.
33. Schmid, A.C., Byrne, R.D., Vilar, R., and Woscholski, R. (2004). Bisperoxovanadium compounds are potent PTEN inhibitors. FEBS Lett. *566*, 35–38.
34. Mustelin, T., Alonso, A., Bottini, N., Huynh, H., Rahmouni, S., Nika, K., Louis-dit-Sully, C., Tautz, L., Togo, S.H., Bruckner, S., Mena-Duran, A.V., and al-Khouri, A.M. (2004). Protein tyrosine phosphatases in T cell physiology. Mol. Immunol. *41*, 687–700.
35. Ferrell, J.E., Jr. (1996). Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem. Sci. *21*, 460–466.
36. Huang, C.Y., and Ferrell, J.E., Jr. (1996). Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA *93*, 10078–10083.
37. Tengholm, A., and Meyer, T. (2002). A PI3-kinase signaling code for insulin-triggered insertion of glucose transporters into the plasma membrane. Curr. Biol. *12*, 1871–1876.
38. Kohler, K., Lellouch, A.C., Vollmer, S., Stoevesandt, O., Hoff, A., Peters, L., Rogl, H., Malissen, B., and Brock, R. (2005).

Chemical inhibitors when timing is critical: a pharmacological concept for the maturation of T cell contacts. ChemBioChem *6*, 152–161.

39. Rogers, B., Decottignies, A., Kolaczkowski, M., Carvajal, E., Balzi, E., and Goffeau, A. (2001). The pleitropic drug ABC transporters from *Saccharomyces cerevisiae*. J. Mol. Microbiol. Biotechnol. *3*, 207–214.
40. Stein, W.D. (1998). Kinetics of the P-glycoprotein, the multidrug transporter. Exp. Physiol. *83*, 221–232.
41. Wohlbold, L., van der Kuip, H., Miething, C., Vornlocher, H.P., Knabbe, C., Duyster, J., and Aulitzky, W.E. (2003). Inhibition of bcr-abl gene expression by small interfering RNA sensitizes for imatinib mesylate (STI571). Blood *102*, 2236–2239.
42. Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature *425*, 737–741.
43. Sherman, F. (1991). Guide to Yeast Genetics and Molecular Biology, Volume 194 (San Diego: Academic Press).
44. Bhatt, R.R., and Ferrell, J.E., Jr. (2000). Cloning and characterization of *Xenopus* Rsk2, the predominant p90 Rsk isozyme in oocytes and eggs. J. Biol. Chem. *275*, 32983–32990.
45. Arooz, T., Yam, C.H., Siu, W.Y., Lau, A., Li, K.K., and Poon, R.Y. (2000). On the concentrations of cyclins and cyclin-dependent kinases in extracts of cultured human cells. Biochemistry *39*, 9494–9501.
46. Lipinski, C.A. (2000). Drug-like properties and the causes of poor solubility and poor permeability. J. Pharmacol. Toxicol. Methods *44*, 235–249.
47. Lipinski, C., and Hopkins, A. (2004). Navigating chemical space for biology and medicine. Nature *432*, 855–861.
48. Lipinski, C.A., Lombardo, F., Dominy, B.W., and Feeny, P.J. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. *23*, 3–25.
49. Veber, D.F., Johnson, S.R., Cheng, H.Y., Smith, B.R., Ward, K.W., and Kopple, K.D. (2002). Molecular properties that influence the oral bioavailability of drug candidates. J. Med. Chem. *45*, 2615–2623.
50. Noble, M.E., Endicott, J.A., and Johnson, L.N. (2004). Protein kinase inhibitors: insights into drug design from structure. Science *303*, 1800–1805.
51. McGovern, S.L., Helfand, B.T., Feng, B., and Shoichet, B.K. (2003). A specific mechanism of nonspecific inhibition. J. Med. Chem. *46*, 4265–4272.
52. Ramdas, L., McMurray, J.S., and Budde, R.J. (1994). The degree of inhibition of protein tyrosine kinase activity by tyrphostin 23 and 25 is related to their instability. Cancer Res. *54*, 867–869.
53. Ramdas, L., Obeyesekere, N.U., McMurray, J.S., Gallick, G.E., Seifert, W.E., Jr., and Budde, R.J. (1995). A tyrphostin-derived inhibitor of protein tyrosine kinases: isolation and characterization. Arch. Biochem. Biophys. *323*, 237–242.
54. Ramdas, L., and Budde, R.J. (1998). The instability of polyhydroxylated aromatic protein tyrosine kinase inhibitors in the presence of manganese. Cancer Biochem. Biophys. *16*, 375–385.
55. McGovern, S.L., and Shoichet, B.K. (2003). Kinase inhibitors: not just for kinases anymore. J. Med. Chem. *46*, 1478–1483.
56. Holleran, J.L., Egorin, M.J., Zuhowski, E.G., Parise, R.A., Musser, S.M., and Pan, S.S. (2003). Use of high-performance liquid chromatography to characterize the rapid decomposition of wortmannin in tissue culture media. Anal. Biochem. *323*, 19–25.
57. Duncia, J.V., Santella, J.B., III, Higley, C.A., Pitts, W.J., Wityak, J., Frietze, W.E., Rankin, F.W., Sun, J.H., Earl, R.A., Tabaka, A.C., et al. (1998). MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg. Med. Chem. Lett. *8*, 2839–2844.
58. Rishton, G.M. (2003). Nonleadlikeness and leadlikeness in biochemical screening. Drug Discov. Today *8*, 86–96.
59. Fry, D.W., Bridges, A.J., Denny, W.A., Doherty, A., Greis, K.D., Hicks, J.L., Hook, K.E., Keller, P.R., Leopold, W.R., Loo, J.A., et al. (1998). Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. Proc. Natl. Acad. Sci. USA *95*, 12022–12027.
60. Vieth, M., Higgs, R.E., Robertson, D.H., Shapiro, M., Gragg, E.A., and Hemmerle, H. (2004). Kinomics-structural biology and chemogenomics of kinase inhibitors and targets. Biochim. Biophys. Acta *1697*, 243–257.
61. Yamamoto, N., Takeshita, K., Shichijo, M., Kokubo, T., Sato, M., Nakashima, K., Ishimori, M., Nagai, H., Li, Y.F., Yura, T., et al. (2003). The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino] nicotinamide dihydrochloride (BAY 61–3606) blocks antigen-induced airway inflammation in rodents. J. Pharmacol. Exp. Ther. *306*, 1174–1181.
62. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem. J. *371*, 199–204.
63. Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95–105.
64. Liu, Y., Bishop, A., Witucki, L., Kraybill, B., Shimizu, E., Tsien, J., Ubersax, J., Blethrow, J., Morgan, D.O., and Shokat, K.M. (1999). Structural basis for selective inhibition of Src family kinases by PP1. Chem. Biol. *6*, 671–678.
65. Blencke, S., Zech, B., Engkvist, O., Greff, Z., Orfi, L., Horvath, Z., Keri, G., Ullrich, A., and Daub, H. (2004). Characterization of a conserved structural determinant controlling protein kinase sensitivity to selective inhibitors. Chem. Biol. *11*, 691–701.
66. Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., et al. (2003). An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. Proc. Natl. Acad. Sci. USA *100*, 15434–15439.
67. Frye, S.V. (1999). Structure-activity relationship homology (SARAH): a conceptual framework for drug discovery in the genomic era. Chem. Biol. *6*, R3–R7.
68. Leahy, J.J., Golding, B.T., Griffin, R.J., Hardcastle, I.R., Richardson, C., Rigoreau, L., and Smith, G.C. (2004). Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. Bioorg. Med. Chem. Lett. *14*, 6083–6087.
69. Wissing, J., Godl, K., Brehmer, D., Blencke, S., Weber, M., Habenberger, P., Stein-Gerlach, M., Missio, A., Cotten, M., Muller, S., et al. (2004). Chemical proteomic analysis reveals alternative modes of action for pyrido[2,3-d]pyrimidine kinase inhibitors. Mol. Cell. Proteomics *3*, 1181–1193.
70. Knockaert, M., Gray, N., Damiens, E., Chang, Y.T., Grellier, P., Grant, K., Fergusson, D., Mottram, J., Soete, M., Dubremetz, J.F., et al. (2000). Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. Chem. Biol. *7*, 411–422.
71. Knockaert, M., Wieking, K., Schmitt, S., Leost, M., Grant, K.M., Mottram, J.C., Kunick, C., and Meijer, L. (2002). Intracellular targets of paullones. Identification following affinity purification on immobilized inhibitor. J. Biol. Chem. *277*, 25493–25501.
72. Knockaert, M., Lenormand, P., Gray, N., Schultz, P., Pouyssegur, J., and Meijer, L. (2002). p42/p44 MAPKs are intracellular targets of the CDK inhibitor purvalanol. Oncogene *21*, 6413–6424.
73. Rosania, G.R., Merlie, J., Jr., Gray, N., Chang, Y.T., Schultz, P.G., and Heald, R. (1999). A cyclin-dependent kinase inhibitor inducing cancer cell differentiation: biochemical identification using *Xenopus* egg extracts. Proc. Natl. Acad. Sci. USA *96*, 4797–4802.
74. Brehmer, D., Godl, K., Zech, B., Wissing, J., and Daub, H. (2004). Proteome-wide identification of cellular targets affected by bisindolylmaleimide-type protein kinase C inhibitors. Mol. Cell. Proteomics *3*, 490–500.
75. Wissing, J., Godl, K., Brehmer, D., Blencke, S., Weber, M., Habenberger, P., Stein-Gerlach, M., Missio, A., Cotten, M., Muller, S., et al. (2004). Chemical proteomic analysis reveals alternative modes of action for pyrido[2,3-d]pyrimidine kinase inhibitors. Mol. Cell. Proteomics *3*, 1181–1193.

76. Ding, S., Wu, T.Y., Brinker, A., Peters, E.C., Hur, W., Gray, N.S., and Schultz, P.G. (2003). Synthetic small molecules that control stem cell fate. Proc. Natl. Acad. Sci. USA *100*, 7632–7637.
77. Liu, Y., Shreder, K.R., Gai, W., Corral, S., Ferris, D.K., and Rosenblum, J.S. (2005). Wortmannin, a widely used phosphoinositide 3-kinase inhibitor, also potently inhibits mammalian polo-like kinase. Chem. Biol. *12*, 99–107.
78. Abbott, B.M., and Thompson, P.E. (2004). PDE2 inhibition by the PI3 kinase inhibitor LY294002 and analogues. Bioorg. Med. Chem. Lett. *14*, 2847–2851.
79. Welling, A., Hofmann, F., and Wegener, J.W. (2004). Inhibition of L-type Cav1.2 Ca2+ channels by LY294002 and Go6983. Mol. Pharmacol. *67*, 541–544.
80. Sun, H., Oudit, G.Y., Ramirez, R.J., Costantini, D., and Backx, P.H. (2004). The phosphoinositide 3-kinase inhibitor LY294002 enhances cardiac myocyte contractility via a direct inhibition of Ik,slow currents. Cardiovasc. Res. *62*, 509–520.
81. Pasapera Limon, A.M., Herrera-Munoz, J., Gutierrez-Sagal, R., and Ulloa-Aguirre, A. (2003). The phosphatidylinositol 3-kinase inhibitor LY294002 binds the estrogen receptor and inhibits 17beta-estradiol-induced transcriptional activity of an estrogen sensitive reporter gene. Mol. Cell. Endocrinol. *200*, 199–202.
82. Hajduk, P.J., Bures, M., Praestgaard, J., and Fesik, S.W. (2000). Privileged molecules for protein binding identified from NMR-based screening. J. Med. Chem. *43*, 3443–3447.
83. Aronov, A.M., and Murcko, M.A. (2004). Toward a pharmacophore for kinase frequent hitters. J. Med. Chem. *47*, 5616–5619.
84. Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science *251*, 283–287.
85. Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*, 905–909.
86. Choi, J., Chen, J., Schreiber, S.L., and Clardy, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science *273*, 239–242.
87. Zheng, X.F., Florentino, D., Chen, J., Crabtree, G.R., and Schreiber, S.L. (1995). TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. Cell *82*, 121–130.
88. Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol. *6*, 1122–1128.
89. Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell *10*, 457–468.
90. Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA *92*, 7686–7689.
91. Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. *270*, 27489–27494.
92. Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. *273*, 18623–18632.
93. Sebolt-Leopold, J.S., Dudley, D.T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R.C., Tecle, H., Barrett, S.D., Bridges, A., Przybranowski, S., et al. (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat. Med. *5*, 810–816.
94. Ohren, J.F., Chen, H., Pavlovsky, A., Whitehead, C., Zhang, E., Kuffa, P., Yan, C., McConnell, P., Spessard, C., Banotai, C., et al. (2004). Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. Nat. Struct. Mol. Biol. *11*, 1192–1197.
95. Davidson, W., Frego, L., Peet, G.W., Kroe, R.R., Labadia, M.E., Lukas, S.M., Snow, R.J., Jakes, S., Grygon, C.A., Pargellis, C., et al. (2004). Discovery and characterization of a substrate selective p38alpha inhibitor. Biochemistry *43*, 11658–11671.
96. Martin, J.L., Avkiran, M., Quinlan, R.A., Cohen, P., and Marber, M.S. (2001). Antiischemic effects of SB203580 are mediated through the inhibition of p38alpha mitogen-activated protein kinase: evidence from ectopic expression of an inhibition-resistant kinase. Circ. Res. *89*, 750–752.
97. Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., and Sawyers, C.L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science *293*, 876–880.
98. Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395–401.
99. Jackson, A.L., and Linsley, P.S. (2004). Noise amidst the silence: off-target effects of siRNAs? Trends Genet. *20*, 521–524.
100. Zambrowicz, B.P., and Sands, A.T. (2003). Knockouts model the 100 best-selling drugs—will they model the next 100? Nat. Rev. Drug Discov. *2*, 38–51.
101. Hardy, L.W., and Peet, N.P. (2004). The multiple orthogonal tools approach to define molecular causation in the validation of druggable targets. Drug Discov. Today *9*, 117–126.
102. Chong, Y.P., Mulhern, T.D., Zhu, H.J., Fujita, D.J., Bjorge, J.D., Tantiongco, J.P., Sotirellis, N., Lio, D.S., Scholz, G., and Cheng, H.C. (2004). A novel non-catalytic mechanism employed by the C-terminal Src-homologous kinase to inhibit Src-family kinase activity. J. Biol. Chem. *279*, 20752–20766.
103. Patrucco, E., Notte, A., Barberis, L., Selvetella, G., Maffei, A., Brancaccio, M., Marengo, S., Russo, G., Azzolino, O., Rybalkin, S.D., et al. (2004). PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. Cell *118*, 375–387.
104. Papa, F.R., Zhang, C., Shokat, K., and Walter, P. (2003). Bypassing a kinase activity with an ATP-competitive drug. Science *302*, 1533–1537.
105. Yu, V.P., Baskerville, C., Grunenfelder, B., and Reed, S.I. (2005). A kinase-independent function of Cks1 and Cdk1 in regulation of transcription. Mol. Cell *17*, 145–151.
106. Cohen, P. (2002). Protein kinases—the major drug targets of the twenty-first century? Nat. Rev. Drug Discov. *1*, 309–315.
107. Ortega, S., Prieto, I., Odajima, J., Martin, A., Dubus, P., Sotillo, R., Barbero, J.L., Malumbres, M., and Barbacid, M. (2003). Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat. Genet. *35*, 25–31.
108. Lowell, C.A., Soriano, P., and Varmus, H.E. (1994). Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes Dev. *8*, 387–398.
109. Lowell, C.A., Niwa, M., Soriano, P., and Varmus, H.E. (1996). Deficiency of the Hck and Src tyrosine kinases results in extreme levels of extramedullary hematopoiesis. Blood *87*, 1780–1792.
110. Abeliovich, H., Zhang, C., Dunn, W.A., Jr., Shokat, K.M., and Klionsky, D.J. (2003). Chemical genetic analysis of Apg1 reveals a non-kinase role in the induction of autophagy. Mol. Biol. Cell *14*, 477–490.
111. Weiss, E.L., Bishop, A.C., Shokat, K.M., and Drubin, D.G. (2000). Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. Nat. Cell Biol. *2*, 677–685.
112. Sreenivasan, A., Bishop, A.C., Shokat, K.M., and Kellogg, D.R. (2003). Specific inhibition of Elm1 kinase activity reveals functions required for early G1 events. Mol. Cell. Biol. *23*, 6327–6337.
113. Vanhaesebroeck, B., Rohn, J.L., and Waterfield, M.D. (2004). Gene targeting: attention to detail. Cell *118*, 274–276.
114. Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D., et al. (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science *297*, 1031–1034.
115. Jou, S.T., Carpino, N., Takahashi, Y., Piekorz, R., Chao, J.R.,

Carpino, N., Wang, D., and Ihle, J.N. (2002). Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. Mol. Cell. Biol. *22*, 8580–8591.

116. Wong, S., McLaughlin, J., Cheng, D., Zhang, C., Shokat, K.M., and Witte, O.N. (2004). Sole BCR-ABL inhibition is insufficient to eliminate all myeloproliferative disorder cell populations. Proc. Natl. Acad. Sci. USA *101*, 17456–17461.
117. Liu, Y., Kung, C., Fishburn, J., Ansari, A.Z., Shokat, K.M., and Hahn, S. (2004). Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. Mol. Cell. Biol. *24*, 1721–1735.
118. Williams, M. (2003). Target validation. Curr. Opin. Pharmacol. *3*, 571–577.
119. Fraley, M.E., Hoffman, W.F., Rubino, R.S., Hungate, R.W., Tebben, A.J., Rutledge, R.Z., McFall, R.C., Huckle, W.R., Kendall, R.L., Coll, K.E., et al. (2002). Synthesis and initial SAR studies of 3,6-disubstituted pyrazolo[1,5-a]pyrimidines: a new class of KDR kinase inhibitors. Bioorg. Med. Chem. Lett. *12*, 2767–2770.
120. Bilodeau, M.T., Cunningham, A.M., Koester, T.J., Ciecko, P.A., Coll, K.E., Huckle, W.R., Hungate, R.W., Kendall, R.L., McFall, R.C., Mao, X., et al. (2003). Design and synthesis of 1,5-diarylbenzimidazoles as inhibitors of the VEGF-receptor KDR. Bioorg. Med. Chem. Lett. *13*, 2485–2488.
121. Fraley, M.E., Arrington, K.L., Hambaugh, S.R., Hoffman, W.F., Cunningham, A.M., Young, M.B., Hungate, R.W., Tebben, A.J., Rutledge, R.Z., Kendall, R.L., et al. (2003). Discovery and evaluation of 3-(5-thien-3-ylpyridin-3-yl)-1H-indoles as a novel class of KDR kinase inhibitors. Bioorg. Med. Chem. Lett. *13*, 2973–2976.
122. Manley, P.J., Balitza, A.E., Bilodeau, M.T., Coll, K.E., Hartman, G.D., McFall, R.C., Rickert, K.W., Rodman, L.D., and Thomas, K.A. (2003). 2,4-disubstituted pyrimidines: a novel class of KDR kinase inhibitors. Bioorg. Med. Chem. Lett. *13*, 1673–1677.
123. Murata, T., Shimada, M., Sakakibara, S., Yoshino, T., Kadono, H., Masuda, T., Shimazaki, M., Shintani, T., Fuchikami, K., Sakai, K., et al. (2003). Discovery of novel and selective IKK-beta serine-threonine protein kinase inhibitors. Part 1. Bioorg. Med. Chem. Lett. *13*, 913–918.
124. Murata, T., Shimada, M., Sakakibara, S., Yoshino, T., Masuda, T., Shintani, T., Sato, H., Koriyama, Y., Fukushima, K., Nunami, N., et al. (2004). Synthesis and structure-activity relationships of novel IKK-beta inhibitors. Part 3: orally active anti-inflammatory agents. Bioorg. Med. Chem. Lett. *14*, 4019–4022.
125. Baxter, A., Brough, S., Cooper, A., Floettmann, E., Foster, S., Harding, C., Kettle, J., McInally, T., Martin, C., Mobbs, M., et al. (2004). Hit-to-lead studies: the discovery of potent, orally active, thiophenecarboxamide IKK-2 inhibitors. Bioorg. Med. Chem. Lett. *14*, 2817–2822.
126. Burchat, A.F., Calderwood, D.J., Friedman, M.M., Hirst, G.C., Li, B., Rafferty, P., Ritter, K., and Skinner, B.S. (2002). Pyrazolo[3,4-d]pyrimidines containing an extended 3-substituent as potent inhibitors of Lck—a selectivity insight. Bioorg. Med. Chem. Lett. *12*, 1687–1690.
127. Goldberg, D.R., Butz, T., Cardozo, M.G., Eckner, R.J., Hammach, A., Huang, J., Jakes, S., Kapadia, S., Kashem, M., Lukas, S., et al. (2003). Optimization of 2-phenylaminoimidazo[4,5-h]isoquinolin-9-ones: orally active inhibitors of lck kinase. J. Med. Chem. *46*, 1337–1349.
128. Chen, P., Doweyko, A.M., Norris, D., Gu, H.H., Spergel, S.H., Das, J., Moquin, R.V., Lin, J., Wityak, J., Iwanowicz, E.J., et al. (2004). Imidazoquinoxaline Src-family kinase p56Lck inhibitors: SAR, QSAR, and the discovery of (S)-N-(2-chloro-6-methylphenyl)-2-(3-methyl-1-piperazinyl)imidazo- [1,5-a]pyrido[3,2-e]pyrazin-6-amine (BMS-279700) as a potent and orally active inhibitor with excellent in vivo antiinflammatory activity. J. Med. Chem. *47*, 4517–4529.
129. Das, J., Lin, J., Moquin, R.V., Shen, Z., Spergel, S.H., Wityak, J., Doweyko, A.M., DeFex, H.F., Fang, Q., Pang, S., et al. (2003). Molecular design, synthesis, and structure-Activity relationships leading to the potent and selective p56(lck) inhibitor BMS-243117. Bioorg. Med. Chem. Lett. *13*, 2145–2149.
130. Chen, P., Norris, D., Das, J., Spergel, S.H., Wityak, J., Leith, L., Zhao, R., Chen, B.C., Pitt, S., Pang, S., et al. (2004). Discovery of novel 2-(aminoheteroaryl)-thiazole-5-carboxamides as potent and orally active Src-family kinase p56(Lck) inhibitors. Bioorg. Med. Chem. Lett. *14*, 6061–6066.