The Evolution of Protein Kinase Inhibitors from Antagonists to Agonists of Cellular Signaling

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Abstract

Kinases are highly regulated enzymes with diverse mechanisms controlling their catalytic output. Over time, chemical discovery efforts for kinases have produced ATP-competitive compounds, allosteric regulators, irreversible binders, and highly specific inhibitors. These distinct classes of small molecules have revealed many novel aspects about kinase-mediated signaling, and some have progressed from simple tool compounds into clinically validated therapeutics. This review explores several small-molecule inhibitors for kinases highlighting elaborate mechanisms by which kinase function is modulated. A complete surprise of targeted kinase drug discovery has been the finding of ATP-competitive inhibitors that behave as agonists, rather than antagonists, of their direct kinase target. These studies hint at a connection between ATP-binding site occupancy and networks of communication that are independent of kinase catalysis. Indeed, kinase inhibitors that induce changes in protein localization, protein-protein interactions, and even enhancement of catalytic activity of the target kinase have been found. The relevance of these findings to the therapeutic efficacy of kinase inhibitors and to the future identification of new classes of drug targets is discussed.

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INTRODUCTION

Eukaryotic protein kinases have emerged as key components in most, if not all, signal transduction cascades. The conserved nature of the ATP active sites of protein kinases and the large number of kinases in eukaryotic genomes have made the interrogation of each kinase a challenge to traditional pharmacological methods used to study other enzymes. In spite of these challenges, the number of selective protein kinase inhibitors has exploded in the past ten years largely as a consequence of intense efforts from the pharmaceutical industry to discover inhibitors of kinases dysregulated in cancer and inflammatory disease. Protein kinases have been the topic of a large body of review literature. We point the reader to the following articles pertaining to kinases and disease, inhibitor design, and the analysis of kinases with chemical tools: References 1, 2, and 3, respectively. The aim of this report is (*a*) to explore the various classes of kinase inhibitors, (*b*) provide insight into the mechanistic details about kinase signaling gained through the application of these inhibitors, (*c*) to highlight recent examples where kinase inhibitors have been found to paradoxically stimulate signaling within cells, and (*d*) to discuss the implications of these findings to the future development of kinase-directed therapeutics.

This review begins with the biochemical and structural lessons learned from protein kinase inhibitors that bind in the highly conserved ATP pocket, including the molecular basis

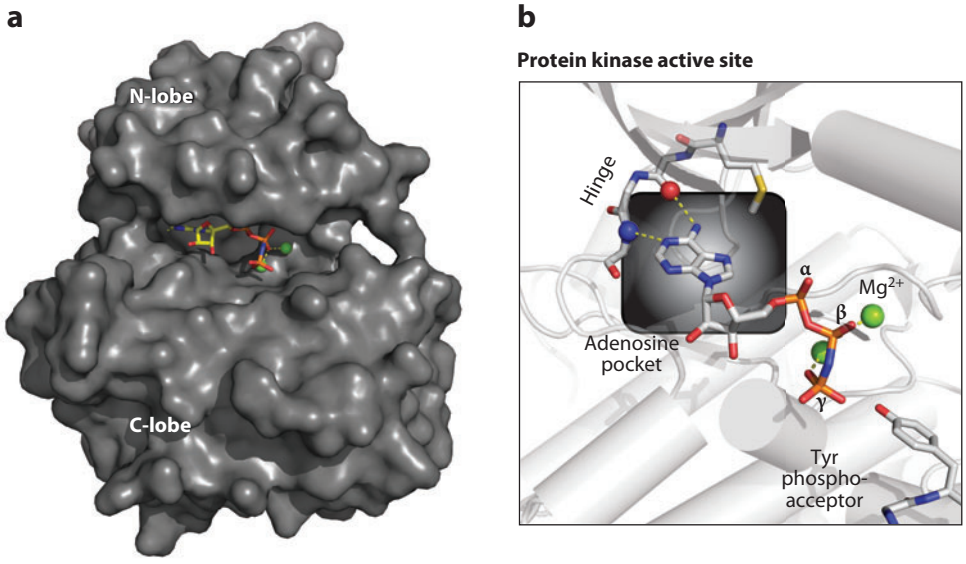


Figure 1

A representative protein kinase ATP substrate complex. Shown is the insulin receptor kinase in complex with ATP and a small peptide substrate (PDB ID 1IR3). (*a*) The ATP-binding pocket within the protein kinase domain lies deep within a cleft bound by the N-terminal lobe, hinge regions, and C-terminal lobe: protein kinase, gray surface; ATP, sticks; Mg^{2+} ions, green spheres. (*b*) Active-site magnification: critical H-bonds between ATP and the main-chain atoms of the hinge are highlighted (*dashed lines*). The γ-phosphate of ATP is poised for transfer to the hydroxyl group of the peptide substrate (in this case, the acceptor residue is tyrosine).

for inhibitor selectivity against closely related kinases. In **Figure 1** we show the structure of a representative protein kinase active site. Although the conserved ATP pocket is where the majority of kinase inhibitors bind, it has long been recognized that targeting pockets outside of the nucleotide region offer a major discrimination filter because the phospho-acceptor pocket and domains outside of the kinase catalytic domain are highly divergent across the family. Examples of such non-ATP competitive kinase inhibitors from natural sources as well as synthetic molecules provide a functional picture of how regions outside of the catalytic site of kinases can regulate kinase function and how next-generation inhibitors may exploit divergent binding pockets.

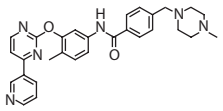
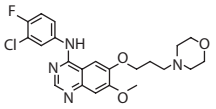
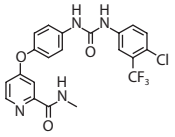
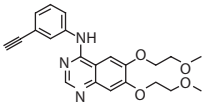
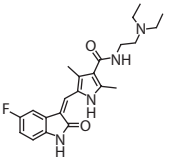
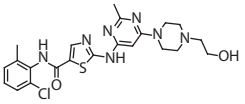
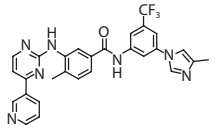
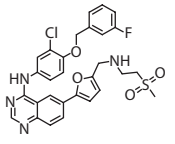
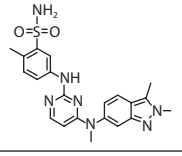
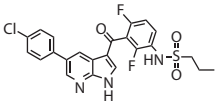
We next consider how kinase inhibitors regulate cellular pathways inside cells. The goal of most kinase inhibitor discovery is to block the pathway mediated by the kinase of interest in cells, animals, and, potentially, patients. Currently nine kinase inhibitors are FDA approved (**Table 1**), providing a wealth of information about how tumors respond to such targeted therapy. The two drugs with the most dramatic success target kinases mutated in particular cancer types: Gleevec, approved nearly 10 years ago to treat BCR-Abl-driven chronic myelogenous leukemia cancers, and PLX4032 (currently in phase II trials), a recent investigational drug, that targets BRAF(V600E) mutant tumors in metastatic melanoma patients. Both drugs show dramatic effects in tumor cells containing somatically mutated activated kinases.

Several recent examples of kinase inhibitors designed initially to antagonize signaling paradoxically induce pathway activation. PLX4032 exhibits completely unexpected activation of BRAF signaling in cells lacking mutated BRAF, which has generated new insights into BRAF signaling while also complicating the clinical

Active site: in kinases, the ATP-binding pocket and region where phosphorylation occurs

Gleevec: (trade name, Imatinib) inhibitor of Bcr-Abl and the first approved kinase inhibitor drug

Table 1

Drug	Structure	FDA approval date	Indication
Imatinib		2001	Chronic myelogenous leukemia Gastrointestinal stromal tumors
Gefitinib		2003	Nonsmall cell lung cancer
Sorafenib		2005	Renal cell carcinoma Hepatocellular carcinoma
Erlotinib		2005	Pancreatic cancer
Sunitinib		2006	Renal cell carcinoma Imatinib-resistant gastrointestinal stromal tumor
Dasatinib		2006	Chronic myelogenous leukemia Philadelphia chromosome positive acute lymphoblastic leukemia
Nilotinib		2007	Imatinib-resistant chronic myelogenous leukemia
Lapatinib		2007	HER2+ breast cancer
Pazopanib		2009	Advanced renal cell carcinoma
PLX4032		Under regulatory review	BRAF mutant metastatic melanoma

development of this investigational drug. Realization that small-molecule kinase inhibitors can actually activate kinase signaling cascades suggests new insights and opportunities for pharmacological manipulation of protein kinase function. Several recent examples of kinase activators have been reported, which significantly expands our understanding of the complexity of kinase regulation and how small molecules can rewire signaling cascades.

The horizon for selective small-molecule kinase inhibitors remains the discovery of compounds that bind outside of the ATP site and can attain high specificity and high potency. An unexplored opportunity for a completely new category of kinase regulator lies in the recent results from cancer genome sequencing, which reveal that a number of kinase mutations that drive oncogenesis inactivate kinase catalytic activity, yet drive cancer. Answering the question of how to inhibit a catalytically dead kinase that causes disease may lead to new therapeutic opportunities.

PROTEIN KINASE INHIBITORS THAT BIND IN THE ATP SITE

Promiscuous Inhibitors

The development of protein kinase inhibitors dates back 25 years beginning with the foundational studies of natural products. These molecules inhibited the activity of the first protein kinases to be assayed biochemically after purification from natural sources. In these early studies the bis-indoylmaleimide staurosporine stood out as a highly potent inhibitor (IC50 = 2.7 nM) of protein kinase C (PKC) that was not competitive with the PKC activator diacylglycerol (4). Recent kinome-wide screening efforts of staurosporine have revealed that this natural product is a potent inhibitor of >90% of kinases (5). Structural studies confirm that this molecule binds in the ATP pocket despite its much larger molecular size (**Figure 2*a***). The staurosporine inhibitor represents the first highly potent inhibitor of protein kinases and demonstrates that, despite the ATP-competitive nature of such ligands, they can out-compete the high cellular concentration of ATP and effectively block kinase function. Structurally, staurosporine revealed the importance of the inhibitor binding in subpockets of the kinase active site that are not occupied by ATP, generating greater affinity than that achieved by the substrate ATP (6). The large size of staurosporine and its broad kinase inhibition suggested that such subpockets were not restricted to particular kinases but that most, if not all, protein kinase family members contained additional pockets for inhibitor binding.

Although staurosporine ushered in the era of kinase inhibitors, its uniform cellular induction of apoptosis, likely due to its promiscuous inhibition of many kinases, made it a poor drug candidate and caused concern that selective kinase inhibition would be unachievable. Other natural products, such as the flavonol quercetin (7), revealed new chemotypes that fit in the ATP pocket, and these led to development of synthetic analogs such as the tyrphostins as inhibitors of receptor tyrosine kinases (8). These early examples progressively narrowed down the spectrum of kinases inhibited by small molecules, although the first truly selective chemotypes eluded rational design and researchers have relied on unbiased cellular- and pathway-based screens.

A Selective Inhibitor of the Map Kinase: P38

In an effort to identify new anti-inflammatory agents, SmithKline Beecham scientists screened for molecules that could block proinflamatory cytokine (IL-1 and TNF) production by monocytes in response to inflammatory stimuli (9). They identified a simple chemotype, the triarylimidazole exemplified by SB202190, that blocked cytokine production. The structure of the molecule did not suggest any known target in the pathway, and thus a search using a radiolabelled analog and a photoaffinity probe was used to identify the novel kinases p38α and p38β as the targets of the hit molecule. Remarkably, the inhibitors are highly selective for these two MAP kinases relative to

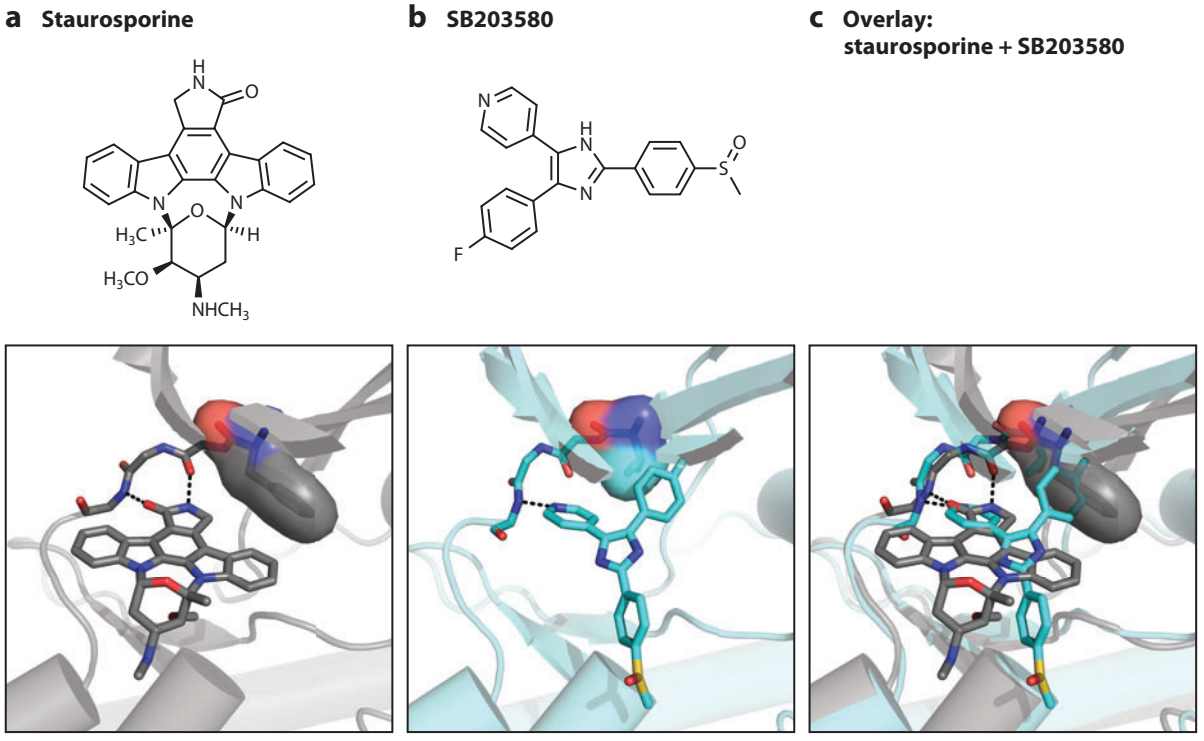


Figure 2

Examples of ATP competitive and reversible inhibitors. (*a*) The natural product and general kinase inhibitor, staurosporine, in complex with CDK2. The gatekeeper phenylalanine residue in CDK2 is shown as a gray surface (PDB ID 1AQ1). (*b*) The pyridinyl imidazole p38 inhibitor SB203580, an analog of SB202190, in complex with the ERK2 mutant Q105T. The gatekeeper mutant threonine residue is shown as a blue surface (PDB ID 1PME). (*c*) The Phe gatekeeper in CDK2 obstructs SB203580 binding. Shown is a structural overlay of both structures from panels *a* and *b*.

the many other MAP kinases. Structural studies and sequence alignments of the map kinase family revealed that a single nonconserved residue in the ATP pocket, now termed the gatekeeper residue, is largely responsible for the high p38α selectivity of the triarylimidazole (**Figure 2*b*,*c***) (10). The gatekeeper residue in p38α is a Thr, whereas in other MAP kinases, such as JNK, the gatekeeper residue is a Met. This provides a selectivity filter for triaryl-imidazole binding to p38 and not JNK, despite their close homology. The gatekeeper residue is a critical determinant of inhibitor selectivity as it is positioned near the N6 amine of ATP in all kinases and can be either a very large amino acid such as Phe or a much smaller residue such as Thr.

Gatekeeper: residue adjacent to N-6 of adenine in the ATP pocket of kinases (corresponds to Thr-338 in c-Src)

The discovery of SB202190 opened the field of kinase inhibitor development to a wealth of drug discovery efforts. Not only did this compound lead to additional inhibitors for p38 such as SB203580 (11), but it also revealed the possibility of finding completely new heterocyclic "drug-like" structures that could distinguish between closely related kinases. The importance of small differences in amino acid identity in kinases could be read out by small molecules allowing for highly selective kinase inhibition. A great wealth of chemical synthesis and screening was pursued, leading to the discovery of potent inhibitors of other kinases such as PP1 for Src family kinases (12) and other Thr gatekeeper kinases including PD 153035, which is directed at EGFR receptor kinases (13). In

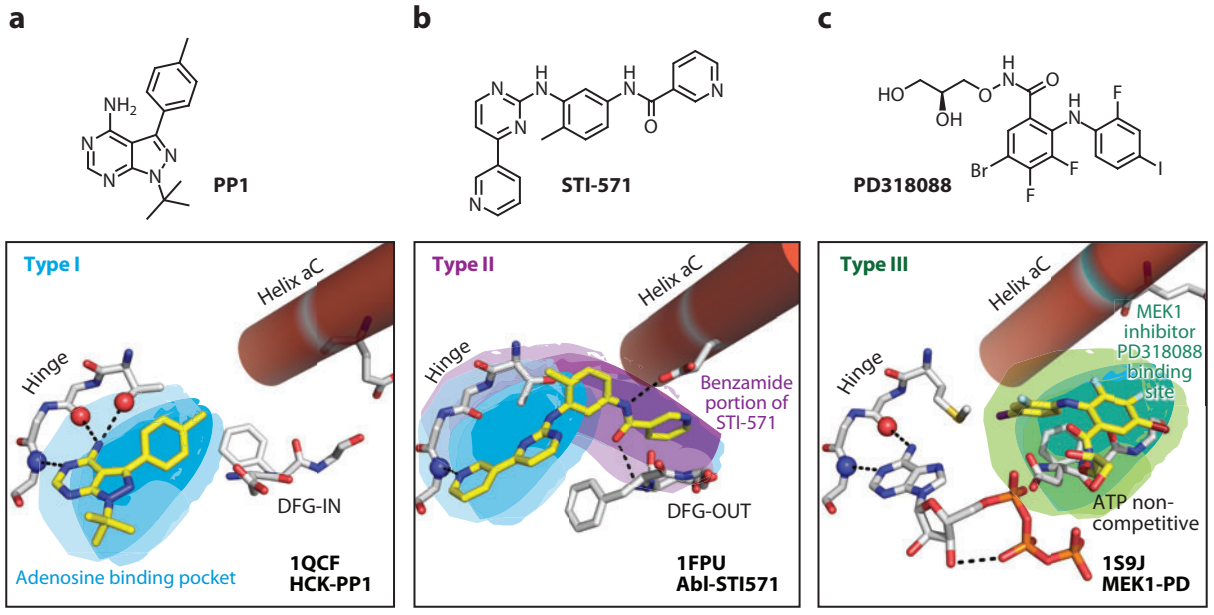


Figure 3

Type I, II, and III kinase inhibitors. (*a*) Type I inhibitors form H-bonds with the kinase hinge and occupy the adenosine binding pocket (*blue*). Type I inhibitors do not require a specific conformation of key structural elements, including helix αC (*red*) or the DFG Mg^{2+}-binding site (*white sticks*), for binding. Shown is the inhibitor PP1 in complex with HCK (PDB ID 1QCF). (*b*) Type II inhibitors occupy the adenosine pocket, but unlike type I binders, they induce a configuration of DFG residues termed DFG-OUT (the D of the DFG flipped 180° relative to the active state conformation). Shown is STI-571 in complex with Abl (PDB ID 1FPU). The benzamide portion of STI-571 (*highlighted purple*) is a common pharmacaphore found in type II binders. (*c*) Type III inhibitors block kinase activity without displacing ATP. Shown is the MEK1 inhibitor PD318088 binding site (*green*) (PDB ID 1S9J).

Figure 3, we show representative type I, II, and III inhibitors, which are described in the following section.

An Unusual MEK Inhibitor that Binds Adjacent to ATP

The high frequency of Ras mutations leading to activation of the Raf-MEK-Erk kinases has directed a great deal of kinase inhibitor discovery efforts for Raf (a MAPKKK), MEK (a MAPKK), and Erk (a MAPK). These three kinases constitute a sequential cascade of kinase substrate relationships. On the basis of this organization, Parke-Davis carried out a pathway-based screen reading out the activity of Erk (14). The inhibitor identified in this screen, PD 098059, was based on a chromone scaffold and was shown to prevent MEK activation of MAPK (15). That PD 098059 could inhibit MEK and not other closely related kinases suggested it was another example of a kinase inhibitor with unique selectivity. The cocrystal structure of MEK1 with PD 318088 (a variant of the core inhibitor) showed surprising simultaneous binding of ATP and the inhibitor (**Figure 3*c***) (16). The inhibitor was bound adjacent to ATP in a pocket partially existing in the MEK1-ATP structure. This was the first inhibitor determined to bind in the kinase active site without making any contacts in common with ATP, now known generally as type III binders.

Conformation-Specific Inhibitors: Gleevec

MEK inhibitors provided some of the first insights into conformational control of kinases by inhibitors and dramatically highlighted the

Type I inhibitor: a small molecule that binds to the active conformation of a kinase in the ATP pocket

Type II inhibitor: a small molecule that binds to the inactive (usually DFG-OUT) conformation of a kinase

Type III inhibitor: a non-ATP competitive kinase inhibitor

DFG-OUT: a conformation of kinases in which the DFG triplet occupies an "out" conformation incapable of complexing Mg in the catalytically essential step of phosphate transfer

potential of pockets adjacent to the nucleotide substrate. The concept that closely related kinases may exhibit similar active conformations yet distinct inactive conformations, providing opportunities for inhibitor selectivity, became a foundation for inhibitor design following the report of the STI-571 (a Gleevec analog) structure in Abl tyrosine kinase. The STI-571 inhibitor exhibits substantial selectivity for Abl over Src. Despite near-identical drug-binding pockets within these two tyrosine kinases, STI-571 inhibits Abl approximately 1000-fold more potently than Src (17), which approaches the selectivity seen in purely biological systems. For example, potassium channels are approximately 10^5 more permeable for K^+ over Na^+ (18). The cocrystal structure of STI-571 bound to Abl revealed a previously unknown conformational change from the active conformation wherein the triplet motif, DFG, shifted 180° to an inactive conformation in a crankshaft-like motion (**Figure 3*b***) (19). That STI-571 required a large conformational change of the target Abl suggested an energetic explanation of its selectivity against Src. If Src were unable to adopt the "DFG-OUT" conformation required by STI-571 binding, then this would explain how kinases with almost identical amino acids in contact with the drug could be differentially inhibited. Presumably, the amino acids in the second sphere surrounding the ATP site controlled such conformational flexibility.

This concept of differential conformational bias between kinases took hold because it offered yet another mechanistic approach for selective inhibitor design. The challenge with this mechanistic approach, however, lies in the lack of structural models that would allow prediction of those kinases capable of adopting particular noncatalytically competent conformations. In practice, many of the unusual conformations of kinases identified structurally have required discovery of selective inhibitors to stabilize the conformation in crystal structure studies (20). In this area, molecular modeling and docking algorithms could prove particularly instrumental for the development of new classes of kinase inhibitors.

The Interplay Between Inhibitor Specificity and Kinase Conformation

Crystallographic analysis of Src in complex with Imatinib revealed Src in the identical DFG-OUT conformation that was observed in the Abl-Imatinib complex (19, 21). The structure suggested that Src could in fact adopt the inactive conformation required for Imatinib binding but could not be used to explain the relatively weak binding affinity between Src and Imatinib.

The paradox could be summarized as follows: Two homologous kinases in identical conformations are bound to the same drug using near-identical drug-binding residues, but one binds with much higher potency to the drug than does the other. To explain the weak binding between Src and Imatinib, researchers suggested the presence of a large energetic barrier inherent to Src that precludes binding to Imatinib with affinity similar to Abl. Efforts to make Src more sensitive to STI-571/Imatinib through point mutations that made Src more Abl-like were largely unsuccessful, further supporting the idea that a conformational bias, distributed through the respective protein kinase domains, must explain the large difference in binding to Imatinib between Abl and Src (21).

A model suggesting that the DFG-OUT conformation was disfavored in Src made a simple testable prediction: No potent DFG-OUT inhibitors of Src should exist. If such DFG-OUT Src binders exist, then Src does not pay a large energetic penalty for adopting this conformation and there must be some other explanation for how Src eludes STI-571 inhibition. In three different studies exploring three distinct chemical series of inhibitors, such DFG-OUT Src binders were reported recently (22–24). These reports overturn one of the key guiding models of selective kinase inhibition. One series of DFG-OUT Src binders, in fact, reveals a distinct contact with a Phe in Src, which is one of the few distinguishable residues between Src (Phe) and Abl(Tyr) (25). Thus, a single hydroxyl group difference facilitates a

closed conformation in the STI-571 structure with Abl, which is less stable in Src. A current formulation of the conformational flexibility basis for inhibitor selectivity appears still to involve amino acid differences between closely related kinases that result in stable conformations that are compatible with drug binding. Importantly, some chemical series contain particular heterocyclic structures that exploit such small differences between kinases, whereas others capable of binding the same conformational states do not distinguish between such small differences. The lessons from STI-571 highlight the interplay between small-molecule chemical series and amino acid differences in kinases and how promiscuity or selectivity can result.

Selective Inhibition by Li^+ Ions

The dominant chemical class of kinase inhibitors are heterocyclic compounds (summarized in **Figure 4**). However, lithium ion is the one remarkably simple inhibitor of the kinase GSK-3β (26). The fact that a metal ion could regulate kinase activity could have been anticipated from the requirement for Mg^{2+} in the ATP-catalyzed reaction. Lithium apparently competes with an active-site Mg^{2+} in GSK-3β, disrupting the catalytic reaction (27). At 500 μM Mg^{2+}, Li^+ (but not K^+) blocks GSK-3β kinase activity with an IC50 of 2 mM (28). The remarkable aspect of the kinase inhibitory activity of Li^+, however, is its selectivity for the kinase GSK-3β, as Li^+ poorly inhibits closely

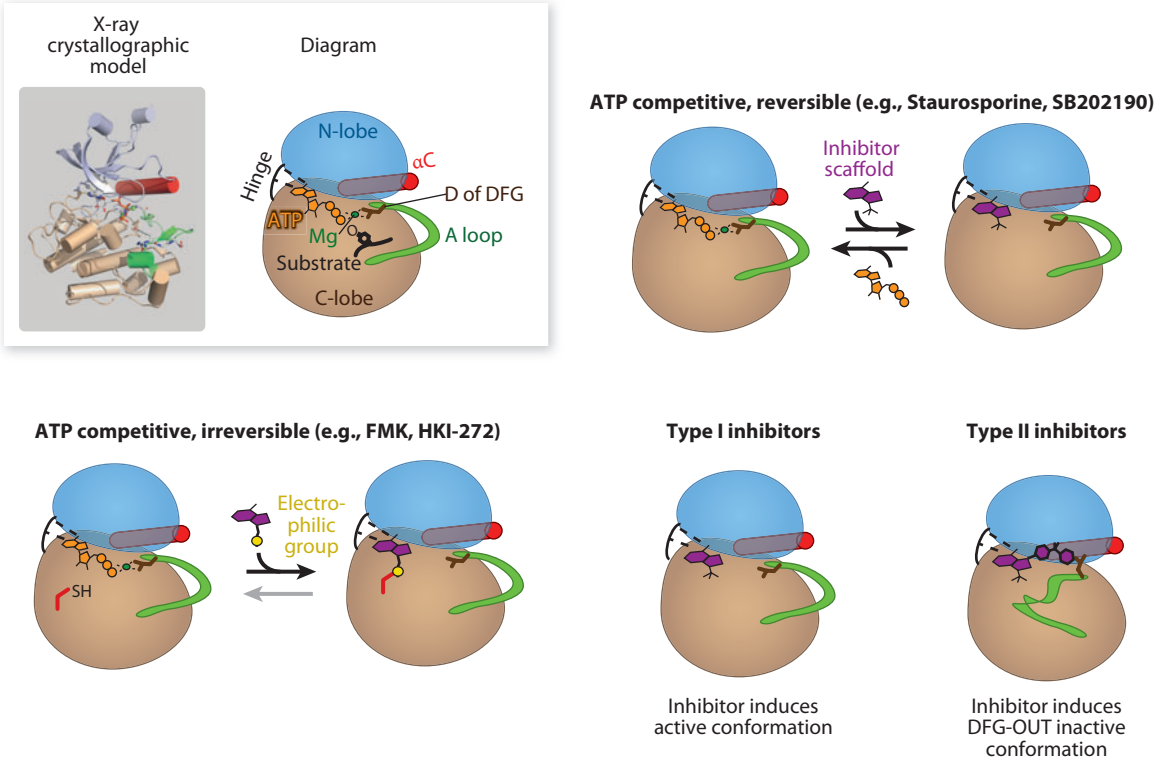


Figure 4

Binders inside the ATP pocket. Summary of active-site modulators: representation of the kinase domain with conserved structural elements highlighted, depicting known mechanisms of protein kinase inhibition. Purple small molecules represent general inhibitor scaffolds (direct the inhibitors to the ATP pocket), and yellow represents an electrophilic group (makes the inhibitors irreversible upon reaction with a Cys in the target kinase).

related kinases. As the only approved drug for treatment of bipolar disorder, Li^+ is an important clinical agent, even though the basis for its efficacy is still not clearly understood. The inhibition of GSK-3β is considered to be one of the prime candidates for the basis of the Li^+ mechanism of action (although GSK-3β is likely not the only or sole target for Li^+) (29, 30).

Chemical Genetic Kinase Inhibitors

Highly selective kinase inhibitors for particular kinases are rapidly becoming available, though the pace of discovery for such important tools is slow compared with the vast size of the protein kinase family (518 human kinases). A method for developing a selective inhibitor for every kinase in the genome that has been established relies on the use of protein engineering to alter the ATP active site of the kinase of interest (31). The altered ATP site is then targeted by a common inhibitor that does not bind to any wild-type enzyme (**Figure 5**). This approach relies on the semiconserved nature of the gatekeeper residue across the yeast, worm, fly, and human kinomes. There are no kinases in these organisms with a small Gly or Ala gatekeeper residue. This natural absence of a small gatekeeper provides a window of selectivity to be introduced at will. Inhibitors such as 1NM-PP1 and 1NA-PP1, which are based on the PP1 series of Thr selective kinase inhibitor, bind only to kinases with an engineered gatekeeper (Gly and/or Ala) and not to the wild-type kinases. Introduction of a Gly gatekeeper kinase in place of the corresponding wild-type kinase into the chromosome produces a cell or organism in which 1NM-PP1 is a monoselective inhibitor

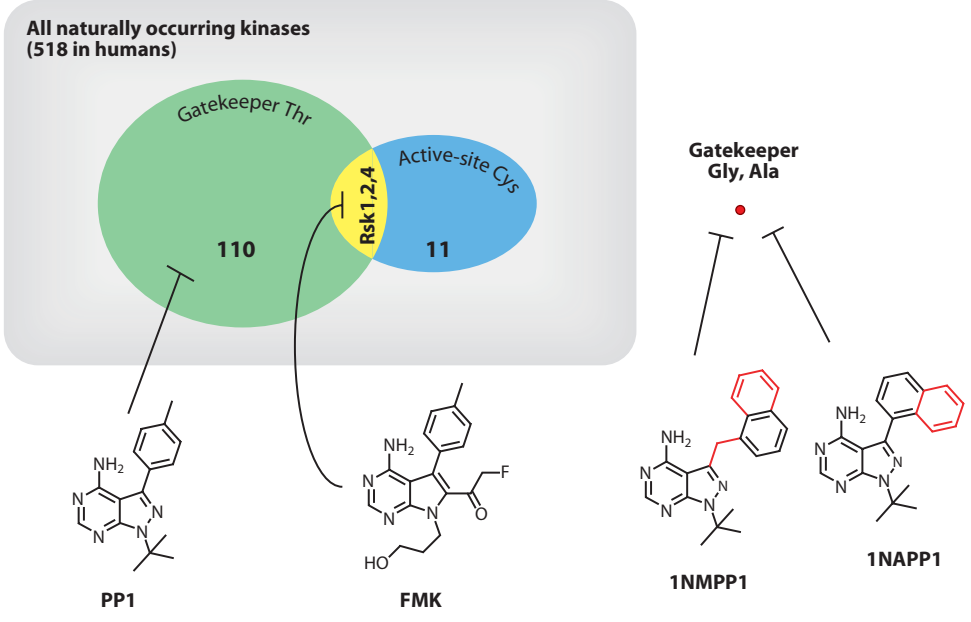


Figure 5

Venn diagram for naturally occurring (*inside gray box*) and engineered kinase mutants (*red dot*). A threonine gatekeeper residue (*green circle*) provides a broad selectivity filter for a subset of kinases that can be targeted by inhibitors such as PP1. Bioinformatic searches identified only three kinases with both a Thr gatekeeper and particular active-site cysteine (Rsk1, Rsk2, Rsk4) (*yellow*), enabling selective inhibition by FMK. Any kinase in gray can be moved into the red circle through mutagenesis of the gatekeeper to a glycine or alanine residue, rendering them sensitive to inhibitors that do not inhibit wild-type kinases, such as 1NMPP1 or 1NAPP1. Red regions of 1NMPP1 and 1NAPP1 fill the space of small gatekeeper pockets (Gly, Ala) but clash with naturally occurring gatekeepers (most often Thr, Phe, or Leu).

of a single kinase of interest. An important control for the specificity of the inhibitor is the parallel analysis of the compound in a wild-type-only cell or organism to ensure that the effects observed are due to inhibition of the kinase of interest. To date, this approach has been applied to more than 50 different kinases in several cell lines including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and mice.

As increasingly more kinase inhibitors are developed and they become used as tools to uncover new cell biology, the question of specificity for the target of interest versus other off-target kinases becomes central. In response to this challenge, a chemical genetic approach has been developed and used to address the specificity of SB203580 as well as an Aurora kinase inhibitor VX-680 (32–34). The method is particularly useful in answering the question of whether a specific cellular effect induced by a particular inhibitor requires inhibition of a particular kinase target. The development of an Aurora A mutant (T217D) resistant to a clinical Aurora inhibitor MLN8237 was used to great effect in deciphering whether the antiproliferative effect of MLN8237 was due to inhibition of Aurora A (34). Expression of drug-resistant Aurora A protected cells from MLN8237, providing strong support that Aurora A and not Aurora B was the key antiproliferative target in the clinic. This experiment also proves that other potential off-targets outside of the Aurora family are not sufficient to block proliferation.

There are several reasons to use the chemical genetic approaches described above for target validation and biological discovery. Chemical genetics is often the first step in rational drug design, as one can assess the influence of modulating a particular target with a specific inhibitor without going through the standard drug discovery process. Techniques such as RNAi, which diminish protein abundance, are often not predictive of therapeutics, which change protein activity (35).

Finding selective inhibitors is a daunting challenge, and nowhere is this more evident than in the protein kinase family. The problem with genetic manipulation is that the changes are irreversible. The problem with using drugs is that they can be nonspecific. Chemical genetics combines the best of both worlds—the selectivity of a genetic perturbation with the control of a pharmacological agent. Many significant discoveries about the mechanism of protein function have been elucidated using chemical genetics (see below).

Structural Bioinformatics–Based Design

Rational design of highly selective kinase inhibitors is remarkably challenging, as the inhibition of GSK-3β by lithium highlights. One could not predict from the structure of GSK3β that it would contain a uniquely sensitive metal binding site compared with other kinases. Despite this challenge, a highly selective inhibitor of the p90RSK kinases was discovered using a structural bioinformatics–based approach (36). A search was carried out looking for two selectivity filters of inhibitor binding across the mammalian kinome (depicted in **Figure 5**). Eleven of the 518 kinases with an active-site cysteine residue that could be targeted by an electrophile bearing inhibitor were identified by sequence alignment. As a second selectivity filter, a small Thr gatekeeper residue was used, which reduced the 11 kinases further to only the p90RSK1,2,4 enzymes. By appending an electrophile onto a Thr gatekeeper–directed kinase scaffold (PP1), a highly p90RSK selective inhibitor, FMK was produced. This combination of reversible and irreversible, cysteine-based inhibitor/kinase recognition has also been exploited effectively in the epidermal growth factor receptor (EGFR) family of kinases (37). It is likely that nonconserved cysteines in kinases will continue to provide important selectivity handles for new inhibitors.

Structural Bioinformatics–Based Targeting of Parasite Kinases

The chemical genetic approach for inhibition of kinases, which relies on mutating the

gatekeeper residue in the kinase of interest to a glycine in the model organism, was never envisioned as a potential drug discovery platform because of the absolute absence of gatekeeper Gly kinases across the kinomes typically studied. The rapid expansion of sequence information from diverse organisms recently revealed that the food-borne parasite *Toxoplasma gondii* contains a single kinase, TgCDPK1, with a natural Gly gatekeeper (38). This essential kinase has a naturally occurring enlarged ATP pocket, making the parasite uniquely sensitive to 3MB-PP1. To prove that TgCDPK1 was the single kinase sensitive to 3MB-PP1, a TgCDPK1Gly->Met parasite was constructed. The mutant was found to be resistant to killing by the compound, confirming the structural bioinformatic prediction of the 3MB-PP1 target within the parasite. This study may provide an entry point into developing 3MB-PP1-like compounds for treatment of parasites that fortuitously contain essential Gly gatekeeper kinases.

KINASE INHIBITORS THAT BIND OUTSIDE OF THE ATP POCKET

Protein-Based Naturally Occurring Selective Kinase Inhibitors

Despite the remarkable selectivity achieved by certain ATP site inhibitors, the ultimate ability to generate a selective inhibitor of every kinase is limited by targeting this widely conserved site. How then can small molecules achieve selective inhibition of each kinase? Are there natural examples of highly selective inhibitors, and if so how do they target a single kinase? The protein substrate binding site of protein kinases is by definition a site of variability between family members because kinases do not phosphorylate a common substrate. Highly selective kinase inhibitors based on the substrate peptide sequence provide important clues as to possibile inhibitors outside of the ATP pocket. Pseudosubstrate sequences with an Ala substituted for the phosphorylatable Ser serve as potent inhibitors of the kinase PKA (**Figure 6*a***) (39). Regions outside of the phosphoacceptor site also provide surfaces or pockets for recognition as exemplified by the CDK-cyclin inhibitor p27Kip1 (40) and the EGFR inhibitor MIG6 (41). A 69-amino acid region of p27 binds to the CDK-cyclin complex and wraps around the N-terminal subdomain of CDK2 continuing to fold into the interface between the two kinase subdomains until a tyrosine hydroxyl group makes H-bond contacts with ATP-interacting residues (**Figure 6*b***). Thus, p27 contains features of ATP and non-ATP site inhibitors.

MIG6 is an endogenous regulator of the EGFR. This receptor and its related family members are critical targets in cancer therapy. Inhibitors, including Lapatinib and Iressa, that target the EGFR active site have found clinical applications in breast and nonsmall cell lung cancers (**Table 1**). Structural analysis of EGFR has demonstrated that the fully ON form of the enzyme is more than a single molecule working alone but rather consists of an asymmetric dimer, in which one protomer (the "activator") induces an active conformation in the second protomer (42). The activator protomer utilizes a surface of its C-lobe to bind to the N-lobe of the second protomer and, in doing so, stabilizes a conformation in which the catalytic efficiency of the second protomer is enhanced (**Figure 6*c***) (42).

Through this unique assembly of kinase domains, the activator of the EGFR dimer plays a role that is strikingly similar to the cyclins, which are known kinase regulators that do not show any structural similarity to a kinase domain (42). Cyclins positively modulate the activity of their CDK partners upon binding to the N-lobe interface. Thus cyclins and EGFR have converged on a similar solution to modulate positively the catalytic output of kinase domain-binding partners.

The finding that one member of the active EGFR dimer does not use catalytic function may explain the prevalence of the catalytically inactive EGF family member HER3. Interestingly, HER3 functions as a positive regulator of EGF signaling (43). More recently, catalytic activity for HER3 has been demonstrated despite several active-site

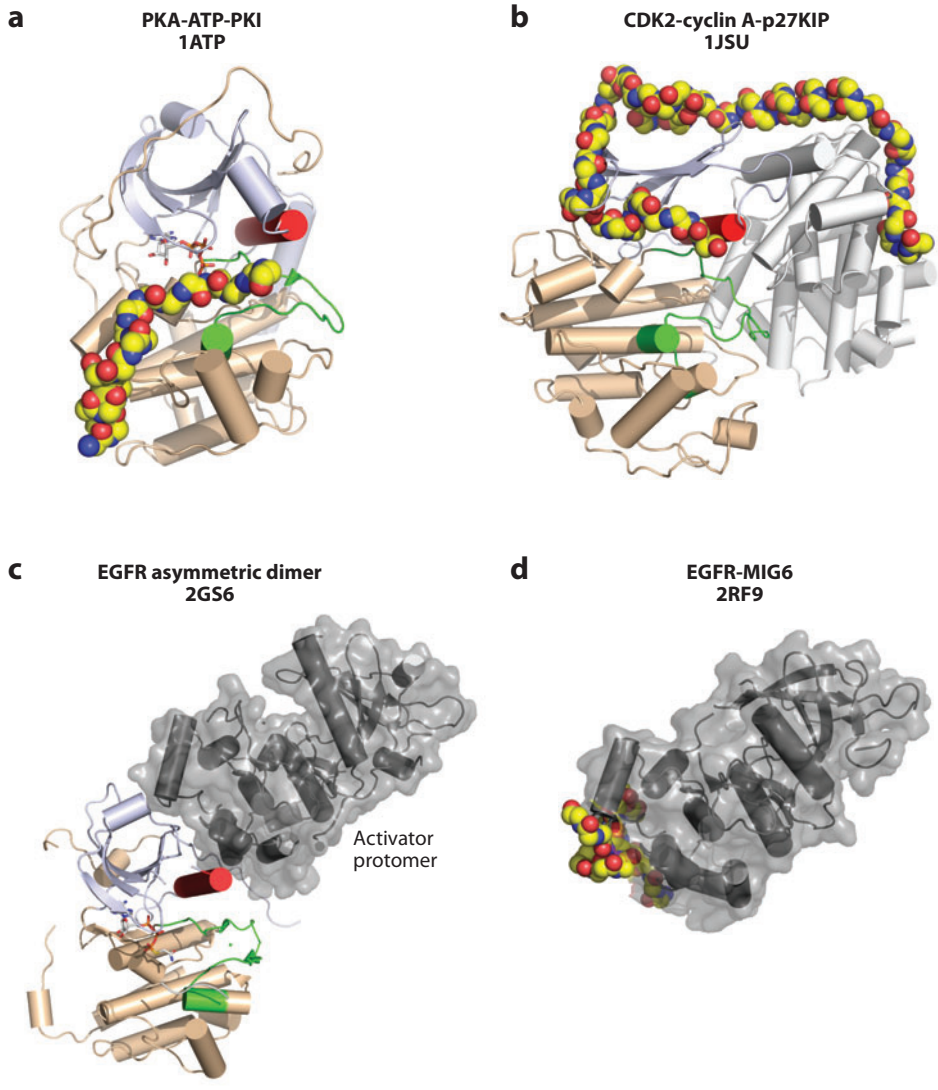


Figure 6

Examples of naturally occurring protein kinase inhibitors. (*a*) The PKA inhibitor PKI (*yellow spheres*) binds the C-lobe of PKA to block substrate binding. (*b*) P27Kip (*yellow spheres*) binds across the CDK-cyclin complex and overlaps partially with the ATP-binding pocket. (*c*) The EGFR asymmetric dimer. In this complex, one EGFR kinase domain (*gray semitransparent surface*) binds and stabilizes an active conformation within a second EGFR kinase domain (*multicolor ribbons*) (PDB ID: 2GS6). (*d*) The inhibitor MIG6 (*yellow spheres*) blocks the formation of the EGFR dimer (PDB ID: 2RF9).

substitutions (44). However, the relevance of this activity still remains undetermined (45). Furthermore, HER3 belongs to a larger subset of all protein kinases termed pseudokinases. It is estimated that approximately 10% of all human kinases are pseudokinases, as they have evolved kinase functions that are independent of catalysis (46). Similar to HER3, pseudokinases may represent true conformational switches, with phosphorylation activity a vestigial remnant hidden within their biological function.

Pseudokinase: members of the kinase family in which one or more of the conserved catalytic residues are lacking

Allosteric site: in kinases, a binding pocket that does not overlap with the ATP active site

The finding that the endogenous EGFR inhibitor, MIG6, blocks the formation of the asymmetric dimer through competition and binding to the C-lobe of the activator protomer (**Figure 6*d***) further confirms the importance of the asymmetric dimer in EGFR regulation. Additional studies will undoubtedly be aimed toward generating synthetic modulators of the EGFR dimerization interface: The biological solutions exemplified by MIG6 and HER3 set the precedent for ones that can act as inhibitors and others that may behave like activators.

These natural peptide–based examples of highly specific kinase inhibitors reveal new binding pockets on kinases for inhibitor discovery. Despite these prevalent natural examples, few small drug-like molecules have been reported to bind to the kinase substrate site, and no molecules with crystallographic characterization are known.

So far we have discussed the chemical tractability of targeting the two substrate pockets found in all kinases. What about allosteric sites outside of these substrate pockets? Kinases serve as highly regulated machines that respond to multiple input stimuli through the regulation of cellular localization, monomer to dimer transitions, and various second-messenger inputs. Small molecules that bind to allosteric pockets have tremendous potential for selective kinase inhibition because these sites are highly divergent across the kinome. Relative to active-site binders, allosteric modulators have proven difficult to find. Most chemical libraries do not contain mimics that match the diversity of biological inhibitors or activators for kinases. Designing allosteric drugs can be very challenging even when crystallographic data are available, whereas the guidelines for making an ATP-competitive drug for a kinase are much better understood. Despite the difficulty in finding allosteric modulators, there are many reasons why they could be extremely useful. By moving outside the highly conserved ATP-binding pocket, target specificity immediately becomes much more facile to achieve. Perhaps more importantly, though, small molecules that bind outside the ATP pocket can differ from ATP-competitive drugs in how they modulate a target and thus can reveal novel or unappreciated aspects about the target and its biological function. The following three examples highlight small-molecule regulators that bind the kinase domain outside the ATP-binding pocket. These small molecules open avenues for novel modes of kinase modulation.

An Allosteric Inhibitor Competes for Myristate

While screening a diverse library of heterocyclic kinase inhibitors, researchers serendipitously found a novel regulator for Bcr-Abl (47): GNF-2 was shown to compete with a myristate lipid binding site on the kinase domain of Abl that is normally engaged through an intramolecular interaction with cAbl's own myristolated N terminus. In cell-culture models, GNF-2 is an extremely selective inhibitor of cells expressing Bcr-Abl, but not of parental cells lacking the oncogene. The crystal structure of an Abl-GNF2-Imatinib complex confirmed a GNF2 binding site within a myristate-binding pocket formed in part through a unique structural element, termed helix αI, found within the Abl kinase domain (**Figure 7*a***) (47). That helix αI forms an integral part of the GNF2 binding pocket could at least partially explain the inhibitor's exquisite selectivity.

In the context of full-length cAbl, engagement of the N-terminal myristoyl modification within the kinase domain functions as a latch for the assembly of the SH2-SH3 units on the kinase domain, maintaining the kinase in an inactive configuration. Remarkably, even in the absence of the inhibitory myristoyl latch that is missing from Bcr-Abl, GNF-2 binding in the kinase domain modulates the conformation of the ATP-binding site within Abl. The connection between the myristate-binding pocket and ATP-binding site was further supported by the finding that GNF2 binding within Abl could increase the sensitivity of Abl to ATP-competitive inhibitors (47). The pathway for

coupling the distinct myristoyl- and ATP-binding pockets likely arose as part of Abl's own autoregulatory mechanism. GNF2 shows that this communication network can be co-opted through a small-molecule drug.

An Interdomain Inhibitor of AKT

The assay for identifying a modulator of a target can be critically important. GNF2 would not have been identified as a "hit" for Abl in the same way as Imatinib if a recombinant form of the purified Abl kinase domain was utilized in the initial screen. The same could be said for the allosteric inhibitor termed AKTi, which was originally identified by Merck scientists (48). This compound emerged from a library of 270,000 molecules as a potent inhibitor of an AKT construct representing the native protein and containing the kinase domain of AKT linked directly to its regulatory PH domain. AKTi is essentially inactive against AKT fragments consisting of only the kinase domain, suggesting an absolute requirement for the PH domain for AKTi inhibition. While this result may have suggested direct binding of AKTi to the PH domain, more recent biophysical analysis suggests AKTi engages a pocket on the kinase domain of AKT, but one that is regulated through an intramolecular PH domain—kinase domain interaction (49). More recent analysis identified mutations at Trp80 within AKT that block the inhibitory properties of AKTi (50). Recently an improved analog with greater specificity, MK2206, has been reported (51). Future studies will be required to show the binding site for AKTi within a crystallized form of full-length AKT and to determine the structural basis for how AKTi influences AKT activation by upstream regulators, including PDK1.

Kinase Activation via Binding of the PIF Pocket

In the next example, a similar communication pathway between an allosteric site and the ATP pocket is modulated, but here, instead of resulting in kinase inhibition, the small molecule activates its kinase target.

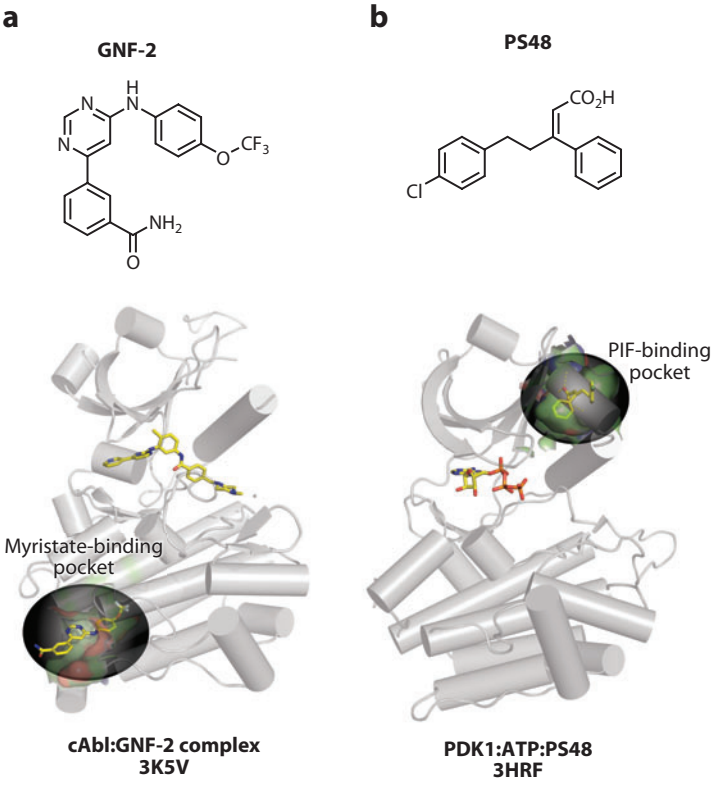


Figure 7

Synthetic modulators that bind within kinase regulatory sites. (*a*) GNF-2 in complex with Abl (PDB ID 3K5). GNF-2 binds within a myristate-binding pocket on the Abl kinase domain to inhibit the enzyme. (*b*) PDK1 in complex with ATP and a hydrophobic motif analog PS48 (PDB ID 3HRF). PS48 binds within the PIF-binding pocket to activate PDK1.

Within the context of the cell, PDK1 activation is complex and involves the action of a number of upstream regulators. The process includes localization to the cell membrane, binding to the PI3K product PIP3, and phosphorylation of the activation loop and a second regulatory site, termed the hydrophobic motif (HM). The HM resides outside the kinase domain within a C-terminal extension and often includes two phenylalanine residues flanking the phospho-site. PDK1, like its own substrate AKT and other members of the AGC kinase family, requires phosphorylation

within the HM for maximal kinase activity. Structural analysis of several AGC kinases has demonstrated that, upon phosphorylation, the HM docks back onto the kinase within a region on the N-lobe termed the PIF pocket. In doing so, the HM stabilizes the active conformation of PDK1. In some instances, the phosphorylated HM comes from the kinase in which it binds, providing a mechanism for kinase autoregulation. In other cases, the HM comes from a substrate, providing a mechanism by which a downstream substrate may activate its upstream kinase.

Based on the understanding of HM regulation for AGC kinases, a computational docking study was initiated leading to the identification of PS48, a small molecule with the features of the HM and containing two phenyl rings bridged by a charged carboxylate functioning as a phospho-mimetic. A recent crystal structure of PS48 bound to PDK1 shows the compound docked within the activating PIF pocket (52) (**Figure 7*b***). In vitro PS48 upregulates PDK1 activity to a level that is similar to a phosphorylated HM, suggesting that the small molecule is a true mimic of PDK1's own regulatory apparatus. Furthermore, through mutational analysis, a residue involved in translating PS48 binding into PDK1 kinase activation was mapped.

It remains to be shown whether PS48 functions in cells. The charged carboxylate of PS48 belies its use as a traditional cellular reagent. However, the discovery of PS48 could lead to future compounds or derivatives that may form the basis for modulating AGC kinases within cells. Very recently a benzoazepoin-2-one was reported as an allosteric regulator of PDK1, also targeting the PIF pocket (53). Reagents such as PS48 could provide a means to identify non-PIF-dependent PDK1 substrates (presumably PS48 would activate PDK1 but compete for PIF-binding substrates). These reagents would also allow activation of PDK1 and possibly bypass the normal upstream signaling components. In doing so, it would be possible to perturb PDK1 function acutely, while leaving other effectors of RTK-Ras-PI3K signaling silent.

IN VITRO INHIBITORS BECOME ACTIVATORS IN CELLS

In the above examples (summarized in **Figure 8**) it is perhaps not surprising that the sites exploited for synthetic ligands are within pockets on the kinase domain that overlap with regions shared by endogenous regulators. GNF2 and PS48 directly mimic the binding interactions of known inhibitors and activators, respectively. What is surprising and could not have been predicted in advance are the small-molecule regulators that repurpose the active site and switch on the signaling properties of a kinase target. The following examples highlight cases in which an ATP-competitive kinase inhibitor in vitro behaves as a positive regulator of kinase function in vivo.

Bypassing the Kinase Activity to Activate an RNase Domain

The unfolded protein response (UPR) pathway is an evolutionarily conserved stress response pathway that matches protein synthesis with protein-folding capacity in the endoplasmic reticulum (ER). The most ancient member of the UPR pathway, Ire1, is a bifunctional kinase-ribonuclease enzyme and a transmembrane protein with an ER lumenal domain that directly senses unfolded proteins. Activation of the unusual RNase domain in Ire1 initiates a nonconventional splicing reaction of an mRNA coding for the transcription factor Hac1 (Xbp1 in mammals) to produce its mature protein and ultimately the UPR. Efforts to study this nonconventional signaling pathway using chemical genetics and by mutation of the Ire1 gatekeeper residue from Leu to Gly produced a 50% loss of Ire1 catalytic function in a cellular assay (54). Paradoxically, addition of the Ire1-Ala mutant specific inhibitor, 1NM-PP1, rescued the lost Ire1 function. Why did addition of the Ire1 inhibitor not block the UPR and further reduce Ire1 function? The loss of Ire1 function from the gatekeeper mutation suggests that the catalytic activity of Ire1's kinase domain is essential for UPR activation. How then could

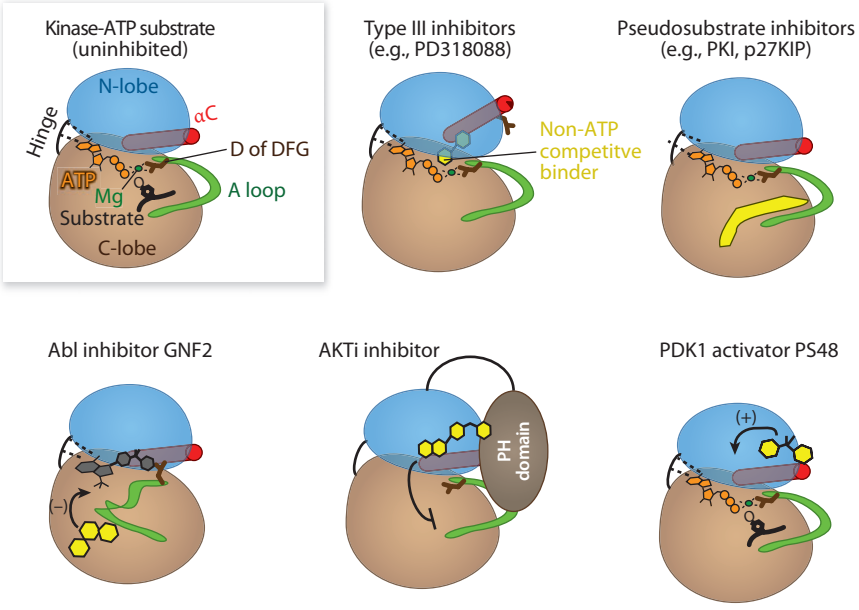


Figure 8

Summary of protein kinase modulators that bind outside the ATP pocket: representation of the kinase domain with conserved structural elements highlighted, depicting the known mechanisms of non-ATP competitive kinase modulation. The non-ATP competitive binders are depicted in yellow.

a kinase inhibitor actually activate a kinase pathway?

Through a number of detailed experiments, the emergent model suggests that, in the case of Ire1, activation of the RNase domain does not require kinase activity per se, but rather can be achieved by simply binding an alternative ligand (an inhibitor) within the ATP pocket of the kinase domain (**Figure 9*a***). Structural data suggest that binding of the inhibitor triggers oligomerization of the receptor into a large ordered helix-like structure that juxtaposes Ire1 kinases, thereby leading to activation of the RNase domain. Why is kinase activity retained through natural selection? If we presume that ATP-site conformation controls the conformation of the kinase domain (evidence supporting this comes from many active and inactive kinase crystal structures), then the cell needs some mechanism to control the affinity of the Ire1 kinase for endogenous ligands such as ADP and ATP (55). The Ire1 kinase transautophosphorylates itself; this could provide the necessary nucleotide-affinity tuning device. Thus, oligomerization by unfolded proteins could provide a mechanism for transautophosphorylation, which then provides for higher occupancy by ATP or ADP, leading to RNAse domain activation. The high-affinity "inhibitor" of Ire1 provides a way to dissociate the intricate mechanism of natural kinase function. The ability of an inhibitor to bypass Ire1 function also provides a new appreciation of kinase functions that can be controlled by developing ligands for the ATP pocket.

Ligand Occupancy of the ATP Site Templates PKC Maturation

A step of kinase regulation that is often overlooked is maturation following synthesis and protein folding but prior to pathway stimulation and full activation of the kinase. The kinase PKCε contains several phosphorylation sites that are required for proper maturation of the enzyme, including the HM

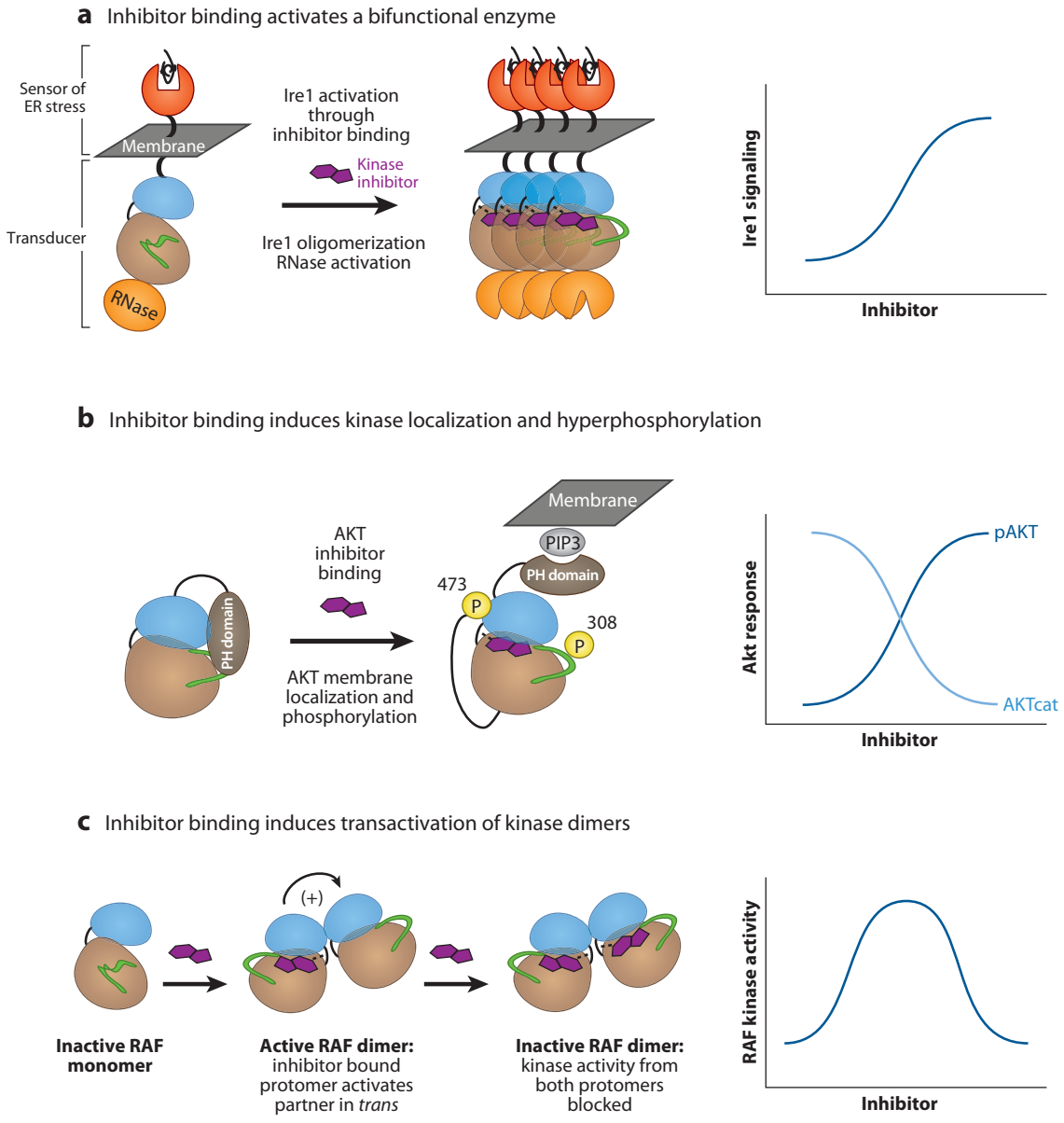


Figure 9

ATP competitive inhibitors as kinase agonists. (*b*) The bifunctional kinase Ire1 contains a cytoplasmic kinase domain linked directly to a ribonuclease (RNase) domain. Inhibitor binding within the kinase domain of Ire1 facilitates RNase activation. The dose dependency of inhibitor displays a sigmoidal relationship with respect to Ire1 signaling. (*b*) The kinase AKT translocates to the membrane of cells to bind the PI3K product PIP3, leading to phosphorylation of two regulatory sites, T308 and S473, which are markers for AKT activation. AKT hyperphosphorylation (pAKT) occurs in an inhibitor-dependent manner upon occupancy of the AKT active site, but leading to reciprocal inhibition of downstream phosphorylation through AKT catalysis (AKTcat). (*c*) The catalytic activity of BRAF is triggered upon binding of two BRAF molecules to form a dimeric structure. Small molecules that bind one BRAF molecule facilitate formation of the dimer, leading to transactivation of the second Raf molecule. High concentrations of inhibitor effectively block signaling from both Raf molecules. Raf displays a bell-shaped dependency on inhibitor concentration within cells, consistent with the model shown to the left of the graph.

site S729, introduced previously in the context of another AGC kinase, PDK1. In the process of studying a kinase-dead mutant of PKCε, K437M, researchers noticed that the mutant did not mature analogously to the wild type as measured by the absence of phosphorylation of HM site S729 (56). This observation had been previously attributed to the need for true kinase autophosphorylation activity. Yet, addition of a general PKC inhibitor related to staurosporine, Bim 1, led to HM phosphorylation, despite the lack of enzyme activity by the inhibitor-occupied kinase—thus challenging the assumption that autophosphorylation activity is required for PKC maturation. Use of an Ala gatekeeper mutant of PKCε and 1NAPP1, allowing for specific inhibition of just PKCε, affirmed the finding with the kinase-dead K437M mutant. The common feature of K437M and M486A was the weakened nucleotide affinity of the mutants owing to partial disruption of the ATP site. The Bim 1 or the more specific 1NAPP1 inhibitor apparently bypasses the need for nucleotide binding during the maturation of PKCε, which results in the enzyme adopting an appropriate conformation for phosphorylation by priming kinases.

Inhibitor Hijacking of AKT Activation

Inhibitor studies with another AGC kinase, AKT, provided an additional example of how an inhibitor could induce an unexpected state of the target kinase. A-443654, a very potent inhibitor of AKT developed at Abbott Labs, blocks phosphorylation in cells of the AKT substrate, GSK-3β (57). Whereas that result was expected, the kinase inhibitor also quite unexpectedly induced hyperphosphorylation of the HM S473 and activation loop T308 phosphorylation of AKT. A chemical genetic system for studying this paradoxical activation revealed, similar to the case with PKCε, that inhibitor binding served to "prime" AKT to adopt the activated state. Because A-443654 is an extremely nonselective inhibitor, a derivatized version of A-443654 (Pr-INDz) was generated that does not bind to any wild-type kinases, but does inhibit Akt containing a gatekeeper Ala mutation. In combination with analog-sensitive alleles of AKT1, -2, and -3 expressed heterologously within cells, Pr-INDz was used to show that inhibitor binding to AKT induced AKT's own hyperphosphorylation within cells. The basis for inhibitor binding in a target leading to hyperphosphorylation of the very same target could be distinguished mechanistically from hyperphosphorylation resulting from inhibition of negative-feedback regulators of AKT. The key experiment was use of a kinase-dead and analog-sensitive allele of AKT that was receptive to inhibitor binding, but by virtue of the kinase-dead mutation could not signal to any AKT substrates. The double AKT mutant was similarly found to become hyperphosphorylated upon addition of Pr-INDz. The experiment effectively ruled out coincidental inhibition of targets other than AKT or feedback upregulation as the basis for AKT hyperphosphorylation. The chemical genetic studies suggested that A-443654 likely also leads to upregulation of endogenous AKT phosphorylation through a similar mechanism.

In the case of inhibitor activation of AKT priming, the kinases responsible for AKT phosphorylation are localized at the cell membrane, whereas the unprimed AKT kinase is cytosolic. During normal growth-factor stimulation, the cytosolic AKT is localized to the cell membrane via its PH-domain binding of PIP3, produced by PI3K. Yet, how did AKT come to be phosphorylated by membrane-bound kinases TORC2 and PDK1, even in the absence of PI3K activation and growth factor stimulation? Immunofluorescence studies of AKT following A-443654 treatment revealed that the inhibitor induced AKT membrane localization and that addition of a PI3K inhibitor abrogated this drug-induced membrane localization (58). Thus, the inhibitor of AKT induced a rearrangement of the AKT PH domain to increase its affinity sufficiently so as to bind the low basal level of PIP3 at the membrane (**Figure 9*b***).

This form of inhibitor-induced AKT priming was referred to as inhibitor hijacking to denote the ability of the inhibitor to bypass the need for complex growth-factor activation of multiple enzymes to produce doubly phosphorylated AKT308/473PP.

In the PKCε and AKT cases, the binding of a ligand to the ATP site leads to hyperphosphorylation of the kinases on the natural priming sites necessary for protein stability and activity. The biochemical basis for such an effect could come from enhancement of upstream kinase activity or a protection from phosphatase-mediated dephosphorylation. Support for the latter mechanism comes from in vitro studies of dephosphorylation of PKCδ where the presence of ATP-Mg^{2+} greatly suppressed protein phosphatase 2A–mediated dephosphorylation (59). Further support for this mechanism comes from the rapid loss of AKT hyperphosphorylation upon inhibitor wash-out from cells. Thus, both in vitro and in vivo experiments seem to suggest ligand binding in the ATP pocket protects kinase phosphorylation sites from dephosphorylation (58). The interplay of phosphatases and kinases in controlling the steady-state level of specific phosphorylation events is necessary to truly understand the bases for many highly regulated phosphorylation events in cells. The absence of specific phosphatase inhibitors makes these studies currently difficult.

The preceding examples of ligand-based modulation of Ire1-RNase activity and PKC/AKT drug-induced hyperphosphorylation have one important feature in common: The kinase ligand inhibits kinase activity. This seems completely self-evident because the ligand competes for the ATP site, preventing the phosphodonor from binding to the active site. In fact, it seems impossible to imagine any case in which an ATP-competitive kinase inhibitor could enhance substrate phosphorylation. An unexpected observation from cellular studies with RAF kinase inhibitors revealed this could actually be occurring, although determination of the mechanism required almost 10 years to be uncovered (60).

A RAF Inhibitor That Stimulates RAF Activity

RAF phosphorylates and activates MEK. RAF has been an attractive and important drug target because it is mutated in 6% of all cancers and is immediately downstream of K-RAS, the most frequently mutated oncogene in cancer. Many different chemical series of RAF inhibitors have been developed with a clinically approved drug sorafenib (Nexavar). In addition, a preclinical compound, PLX4720, provides good benchmarks. The three isoforms of RAF are A-, B-, and C-RAF, which are all targeted by current RAF inhibitors. Cancer mutations occur in B-RAF; V600E is the predominant form in melanoma. In V600E B-RAF mutant cells, PLX4720 is a potent inhibitor of MEK phosphorylation. As a result of this potent inhibition, a clinical RAF inhibitor related in structure to PLX4720, PLX4032, has shown tumor regression in 90% of patients with BRAF(V600E) mutation (61). In studies of cells with other pathway-activating mutations, such as K-RASG12V, but with wild-type BRAF kinase, the effect of the Raf inhibitors is paradoxically to activate MEK phosphorylation. This is an intriguing observation first made in 1999 by Hall-Jackson et al. (60), although at that time no mechanism was uncovered. Strikingly, during the clinical trial of PLX4032, approximately one-third of melanoma patients treated at the maximum tolerated dose of PLX4032 developed a different cancer, squamous cell carcinoma (61). The mechanistic basis for RAF-inhibitor-induced MEK phosphorylation has implications for understanding not only RAS-RAF-MEK signaling, but also the drug-induced development of secondary cancer in patients.

The key observation relating to RAF-inhibitor-induced MEK phosphorylation was the bell-shaped dose-response curve, such that maximal stimulation occurred at intermediate doses and the activation could be suppressed at high dose (62, 63). A model consistent with these data is one in which the inhibitor binding to a RAF monomer induces RAF dimerization

(**Figure 9*c***). In the dimeric state, with one RAF protomer containing the inhibitor and the other protomer remaining inhibitor free, the RAF kinase activity is significantly above the level achieved by the monomeric form of the kinase. At the high level of inhibitor concentration when both protomers of the RAF dimer are occupied by inhibitor, the activity is completely inhibited, which would explain the high-dose suppression of activation. Other models that could explain the phenomenon involve negative feedback between RAF isoforms (64). If BRAF activity negatively regulates CRAF phosphorylation of MEK, then a BRAF-selective inhibitor would lead to activation of CRAF and an increase in MEK phosphorylation.

To distinguish between these models, it was necessary to develop completely isoform-selective inhibitors of RAF using chemical genetics, because the majority of RAF inhibitors were nonselective between B- and C-RAF isoforms. By introduction of a nonconserved Cys residue into the CRAF kinase it was possible to develop a selective inhibitor of just CRAFS248C (62). To distinguish between models in which a negative-feedback loop existed and one in which the inhibitor induced dimerization and activation of the primary target of the inhibitor, a kinase-dead version of CRAF was used. Cells expressing a kinase-dead/inhibitor-sensitive allele of CRAF (S248C/D486N) showed no MEK phosphorylation until the addition of an allele-specific inhibitor induced dimerization with wild-type CRAF (drug insensitive) and increased MEK phosphorylation (62). As proof of the requirement for dimerization, a mutation known to disrupt RAF-RAF dimerization, R401A, was shown to prevent the drug-induced activation of MEK phosphorylation in this system. These studies suggest that a small-molecule inhibitor of a kinase that is regulated by a monomer-dimer transition can actually be activated by the inhibitor (62). Specifically for the RAF inhibitor case, these studies highlight the striking difference in signaling between a BRAFV600E cell (MEK inhibited) versus a wild-type BRAF cell (MEK activated) by the BRAF inhibitors. The clinical implications of these findings for patients with KRAS- or BRAF-driven tumors will produce one of the major areas of clinical and basic research in molecularly targeted therapy in the coming decade.

Kinase-Inactivating Mutations and Cancer

If kinase inhibitors can induce signaling by inducing conformational states that transactivate inhibitor-free kinase molecules in cells, it seems plausible that some somatic mutations in kinases driving cancer may paradoxically inactivate the catalytic activity of a kinase. In kinome-wide unbiased screens for kinase mutations in cancer, a large number of such inactivating mutations have been uncovered (**Figure 10**) (65). For example, in the mutant forms of DAPK3, HCK, and LYN, the conserved Mg^{2+}-binding aspartate that is required for catalysis has been found mutated to residues that are not negatively charged and thus cannot chelate the essential Mg^{2+}. This has also been observed in BRAF where kinase-inactivating mutations are known to be driver mutations and inactivate the same aspartate BRAF D593V (66). Much work still needs to be done to demonstrate that such somatic mutants are indeed driver mutations and not merely passengers induced by genomic instability with no benefit to the cancer cell.

A challenge for drug discovery is understanding how to create a drug for an inactive kinase that may cause cancer. At first glance, it makes no sense to inhibit a dead enzyme to stop signal transduction. However, if the mutationally inactivated kinase signals by binding to an active wild-type kinase that carries out the catalytic function, then two drug strategies emerge. First, an inhibitor of the mutationally activated kinase will also inhibit the wild-type kinase, thus blocking the pathway. In this case, the binding of the inhibitor to the inactive mutant kinase is not relevant to the activity of the inhibitor. Second, an inhibitor of the mutant (dead) kinase may be designed to lock the kinase in a state that is not compatible with transactivation of the wild-type enzyme.

P-loop mutations

		Mutation	Predicted catalytic activity
BRAF	R I G S G S F G T V	G469A	Inactive
MLCK	V L G G G R F G Q V	G601E	Inactive
AURKC	P L G K G K F G N V	G18E	Inactive
MAP3K10	I I G V G G F G K V	G107E	Inactive
STK38L	V I G R G A F G E V	G99A	Inactive
STK32B	A I G K G S F G K V	G35E	Inactive
MST4	R I G K G S F G E V	G36W	Inactive
	G x G x x G		

Activation segment mutations

		Mutation	Predicted activity
BRAF	D F G L A T V K - - - - - - - - - - - - S R W S G S H Q - - F E Q L - - S G - - - S I L W M A P E	D594V	Inactive
DAPK3	D F G I A H - - - - - - - - - - - - K I E A G N - - - - - E F K N I F G - - - T P E F V A P E	D161N	Inactive
HCK	D F G L A R - - - - - - - - - - - - V I E D N - - - - - E Y T A R E G A K F P I K W T A P E	D378G	Inactive
LYN	D F G L A R - - - - - - - - - - - - V I E D N - - - - - E Y T A R E G A K F P I K W T A P E	D385Y	Inactive
FYN	D F G L A R - - - - - - - - - - - - L I E D N - - - - - E Y T A R Q G A K F P I K W T A P E	G410R	Inactive
EPHA3	D F G L S R - - - - - - - - - - - - V L E D D P E A - - - A Y T T R - G G K I P I R W T S P E	G766E	Inactive
MAPK8	D F G L A R T - - - - - - - - - - - A G T S F M M T - - - P Y V V T - - - - - - R Y Y R A P E	G171S	Inactive
CDK11	D M G F A R L F - - - - - - - - - - N S P L K P L A D L D P V V V T - - - - - - F W Y R A P E	G175S	Inactive
MAST205	D F G L S K M G L M S L T T N L Y E G H I E K D A R E - - F L D K Q V C G - - - T P E Y I A P E	G655A	Inactive
SgK494	D F G L S R - - - - - - - - - - - - H V P Q G A - - - - - Q A Y T I C G - - - T L Q Y M A P E	R291C	?
CDKL2	D F G F A R T L - - - - - - - - - - A A P G E V Y T - - - D Y V A T - - - - - - R W Y R A P E	R149Q	?
PSKH2	D F G L A - - - - - - - - - - Y S G K K S G D W - - - - - T M K T L C G - - - T P E Y I A P E	K212I	?
PRKCB1	D F G M C K - - - - - - - - - - E - N I W D G V - - - - - T T K T F C G - - - T P D Y I A P E	V496M	?
CDK8	D M G F A R L F - - - - - - - - - N S P L K P L A D L D P V V V T - - - - - - F W Y R A P E	D189N	?
KSR2	D F G L F S I S G - - - - - - - - - V L Q A G R R E - - D K L R I Q N G - - - W L C H L A P E	R855H	?
FGFR3	D F G L A R - - - - - - - - - - - - D V H - N L D - - - - Y Y K K T T N G R L P V K W M A P E	K650E	?
DYRK1B	D F G S S C - - - - - - - - - - - - Q L G Q R I Y - - - - Q Y I Q S - - - - - - - R F Y R S P E	Q275R	?
PAK7	D F G F C A - - - - - - - - - - - - Q V S K E V P - - - - K R K S L V G - - - T P Y W M A P E	V604I	?
EPHA2	D F G L S R - - - - - - - - - - - - V L E D D P E A - - - T Y T T S - G G K I P I R W T A P E	G777S	?
FLT1	D F G L A R - - - - - - - - - - - - D I Y K N P D - - - - Y V R K G D T - R L P L K W M A P E	L1061V	?
PAK3	D F G F C A - - - - - - - - - - - - Q I T P E Q S - - - - K R S T M V G - - - T P Y W M A P E	T425S	?
FGFR1	D F G L A R - - - - - - - - - - - - D I H - H I D - - - - Y Y K K T T N G R L P V K W M A P E	V664L	?
SGK2	D F G L C K - - - - - - - - - - - E G - V E P E D - - - - - T T S T F C G - - - T P E Y L A P E	E259K	?
NTRK3	D F G M S R - - - - - - - - - - - - D V Y - S T D - - - - Y Y R V G G H T M L P I R W M P P E	R721F	?
Wee1b	D L G H A T - - - - - - - - - - - - S I N K P - - - - - - K V E E G - - - - - D S R F L A N E	R398H	?
PTK2	D F G L S R - - - - - - - - - - - - Y M E D S - - - - - - T Y Y K A S K G K L P I K W M A P E	A612V	?
ALS2CR7	D F G L A R A K - - - - - - - - - - S I P S Q T Y S - - S E V V T L W Y R P P D A L L G A T E	E225D	?
	D F G		
	A P E		

Figure 10

An alignment of kinases found mutated in various cancers. Somatic mutations found within protein kinases from diverse human cancers are listed to the right of the sequences. For BRAF, in which multiple mutations have been found, the listed mutant refers to a substitution at the first position highlighted. Mutations predicted to diminish kinase catalytic function are listed. The P loop, which is important for the coordination of ATP phosphates and catalysis (consensus GXGXXG), and the activation segment, which includes Mg-coordinating residues and spans the DFG and APE motifs, are shown. Yellow highlights mutations at residues that are conserved and required for phosphorylation. Blue highlights mutations at nonconserved residues. The figure is adapted by permission from Macmillan Publishers Ltd., *Nature*, copyright 2007 (65).

This is a plausible strategy using the various type II and type III kinase inhibitors. Importantly, such conformation-specific kinase inhibitors were originally designed to overcome the challenge of achieving high selectivity, but now they may be used as specific inducers of inactive conformations in their targets. The use of such different classes of inhibitors to induce different cellular conformations of kinases would be predicted to have profoundly different signaling outputs. The broader implication of these principles is the transition in thinking of kinase inhibitors as pure antagonists of kinases and the appreciation that there can be ATP-site binders that are agonists of kinase signaling. By applying such principles to kinase drug discovery there may be new opportunities for developing drugs that have improved activity and enhanced therapeutic indices for treating a wide variety of diseases in which kinases are involved.

It is intriguing that a large proportion of mutant kinases identified in cancer genomes, as well as many naturally occurring kinases, lack essential conserved catalytic residues. The occurrence of these mutations point to the ATP pocket as an important site of regulation. Future efforts will involve investigating the properties of these atypical mutant kinases in greater detail (67). Are they truly inactive? Do they bind ATP? Have they evolved distinct mechanisms for catalyzing phospho-transfer? Can their function be manipulated with small-molecule ligands? Recent structural analyses of pseudokinases have revealed intriguing modes of regulation (45, 68–70) and catalysis (44, 71). As putative regulators of cell signaling networks (72, 73), chemical discovery efforts for mutant pseudokinases promise to be rich with new breakthroughs in this burgeoning area of kinase research.

SUMMARY

The high frequency of kinase mutations in driving various cancers and the prominence of kinases in immune cell signaling have brought kinases to the front of drug discovery efforts in cancer and autoimmune disease treatments. This attention has brought a wealth of chemical discovery yielding highly diverse drug-like ligands that target multiple conformations and surfaces of kinases. As these inhibitors are used in cells, model organisms, and patients, researchers have gained a wealth of information about kinase signaling that was largely invisible to genetic approaches for the study of kinases. As the field progresses we will likely see advances in the exploitation of conformation-specific inhibitors and paradoxical activators of kinase signaling to tailor new medicines. There may be a limit to the number of single-kinase drug targets that can be effectively inhibited to achieve patient responses. The highly degenerate and adaptable kinase networks in disease cells pose a challenge to highly selective inhibitors because cells can dynamically adapt to the presence of such molecules. Another frontier for kinase inhibitor development is rational polypharmacology in which small molecules with specific combinations of protein kinase targets or protein and lipid kinase targets are developed (74, 75). Such designed molecules may exhibit enhanced efficacy and less toxicity if they match the spectrum of kinases involved in disease cells and minimally perturb normal cell signaling.

Agonist: kinase ligand that activates kinase activity

Antagonist: kinase inhibitor that blocks kinase activity

SUMMARY POINTS

1. Protein kinases are the critical drivers of many diseases and represent a large class of drug targets.
2. Small-molecule inhibitors of kinases have diverse structures and bases for selective inhibition.

3. ATP-binding inhibitors can be made selective on the basis of nonconserved features of even closely related kinases.
4. The gatekeeper residue is a central determinant of kinase inhibitor selectivity.
5. Kinase inhibitors can paradoxically activate kinases.
6. Kinase inhibitors can activate signaling pathways.
7. Mutations that block kinase catalytic function can trigger signaling pathways in disease. Many kinase-inactivating mutations have been found in cancers and may represent a future class of drug targets.

FUTURE ISSUES

1. Researchers must determine how to design selective kinase inhibitors, inhibitors that bind outside of the ATP site, and inhibitors of mutant kinases that are inactive enzymes.
2. The repertoire of kinase agonists and antagonists for interrogating biological pathways and for generating novel therapeutics must be expanded.
3. New methods in which to assay catalytic and conformational protein kinase properties so that novel modulators can be identified need to be developed.
4. An entire class of kinase inhibitors not discussed in this review include those that derive their efficacy from simultaneously targeting multiple kinases—these have their own challenges but exhibit "emergent" properties in their ability to perturb multiple kinase pathways.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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