Published in final edited form as:

ACS Chem Biol. 2013 December 20; 8(12): . doi:10.1021/cb400663k.

Conformation-selective inhibitors reveal differences in the activation and phosphate-binding loops of the tyrosine kinases Abl and Src

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Abstract

Over the last decade, an increasingly diverse array of potent and selective inhibitors that target the ATP-binding sites of protein kinases have been developed. Many of these inhibitors, like the clinically approved drug imatinib (Gleevec), stabilize a specific catalytically inactive ATP-binding site conformation of their kinases targets. Imatinib is notable in that it is highly selective for its kinase target, Abl, over other closely-related tyrosine kinases, like Src. In addition, imatinib is highly sensitive to the phosphorylation state of Abl's activation loop, which is believed to be a general characteristic of all inhibitors that stabilize a similar inactive ATP-binding site conformation. In this report, we perform a systematic analysis of a diverse series of ATP-competitive inhibitors that stabilize a similar inactive ATP-binding site conformation as imatinib with the tyrosine kinases Src and Abl. In contrast to imatinib, many of these inhibitors have very similar potencies against Src and Abl. Furthermore, only a subset of this class of inhibitors is sensitive to the phosphorylation state of the activation loop of these kinases. In attempting to explain this observation, we have uncovered an unexpected correlation between Abl's activation loop and another flexible active site feature, called the phosphate-binding loop (p-loop). These studies shed light on how imatinib is able to obtain its high target selectivity and reveal how the conformational preference of flexible active site regions can vary between closely related kinases.

INTRODUCTION

Protein kinases are one of the largest protein families in the human genome.(1) These enzymes play important roles in signal transduction networks that control countless intracellular functions, including immunity, morphogenesis, and cell cycle control.(2) Precise control over kinase activity is necessary for proper cellular function. The phosphotransferase activities of protein kinases are mainly regulated on a post-translational level, which is often achieved by modulating the conformation of kinase ATP-binding sites. Due to the necessity of facilitating phosphate transfer, the structural topologies of active kinase ATP-binding sites are highly similar, with key catalytic residues optimally aligned for catalysis.(3) However, freed of the necessity to catalyze phosphate transfer, more variable inactive ATP-binding site conformations are possible.(4)

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SUPPORTING INFORMATION

Supplementary figures and tables, along with detailed material and methods are included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The link between catalytic activity and structure lies in a kinase's internal architecture, which is readily understood through the identification of a network of hydrophobic residues that line the active site and spans both the N-terminal and C-terminal lobes of the catalytic domain. In kinases that are catalytically active, there are two conserved networks of hydrophobic "spines," one regulatory and one catalytic, that line the active site and provide a framework for catalysis (Figure 1A).(5) The necessity of these spines to assemble for catalysis means that essentially only one active kinase conformation exists. Any disruption of either spine gives rise to an "inactive" conformation with reduced catalytic potential.

The regulation of kinase catalytic activity is dependent on the equilibrium between inactive and active ATP-binding site conformations. The dynamic nature of kinase active sites makes studying specific conformations challenging, but small molecule inhibitors that stabilize specific inactive forms have aided this study. One of these conformations is exemplified by the interaction of Abl with imatinib (Gleevec) (Figure 1B).(6) Like many other kinases, Abl has an activation loop that contains one or more residues that increase catalytic activity upon phosphorylation. At the base of the activation loop is an Asp-Phe-Gly (DFG) motif that is highly conserved across the protein kinase family.(3) Imatinib is an example of a type II kinase inhibitor, wherein the activation loop must undergo a dramatic conformational change that "flips" the DFG motif aspartate residue away from the active site, and projects the phenylalanine residue into the ATP-binding site (DFG-out conformation), in order to accommodate drug binding. Since the phenylalanine in the DFG motif is a key component of one of Abl's hydrophobic spines, its translocation has both structural and functional consequences: structurally, it severs the regulatory spine by uncoupling the N-lobe from the C-lobe, and functionally, it displaces the DFG motif's conserved catalytic aspartate from the ATP-binding pocket. At first, the exceptional selectivity of imatinib for Abl over other closely-related kinases was thought to be due to Abl's rare ability to adopt the DFG-out conformation. However, over the last decade a number of diverse kinases, including the tyrosine kinase Src, have been structurally characterized in the DFG-out conformation using a host of different type II inhibitors.(7, 8) Abl and other closely-related kinases have also been characterized in an additional inactive conformation commonly known as "CDK-like" inactive.(9) In this conformation, the DFG motif phenylalanine remains aligned with the rest of the regulatory spine, but instead helix α C is rotated and translocated away from the active site (Figure 1C). This motion also disrupts the position of a catalytic residue also on helix α C. Small-molecule ligands that stabilize the CDK-like inactive form have also proven to be useful for characterizing this conformation.(10-12)

The interaction of imatinib with Abl is also one of the best characterized examples of activation state-selective inhibition.(13) Phosphorylation of the activation loop residue Tyr412 (pY412) increases the catalytic activity of Abl by ten-fold.(14) Since the DFG motif is only twelve residues upstream from Tyr412, it is believed that this phosphorylation event enhances catalysis by stabilizing the active conformation (DFG-in) of the activation loop. It has been reported that pY412 Abl is more than two orders of magnitude less sensitive to inhibition by imatinib than the unphosphorylated form (npY412).(15) Therefore, it is reasonable to conclude that a kinase stabilized in the active conformation through phosphorylation would pay an additional energetic penalty in adopting the DFG-out inactive conformation. Moreover, imatinib is not the only type II inhibitor of Abl that exhibits this effect; AST-487 is a >30-fold more potent inhibitor of unphosphorylated Abl than the phosphorylated form.(13, 16)

However, the mitogen activated protein kinase (MAPK) p38 α , which is one of the only other kinases where the phospho-dependence of type II inhibition has been extensively characterized, shows no such relationship between activation loop phosphorylation and type II inhibitor sensitivity. Like Abl, the catalytic activity of p38 α is increased when its

activation loop is phosphorylated. Indeed, dual phosphorylation of the threonine and tyrosine residues on the TGY motif in the activation loop results in an increase in catalytic activity of several orders of magnitude.(17) Also analogous to Abl, p38 α is inhibited by a number of potent type II ligands that stabilize the DFG-out conformation.(18-20) For example, the selective type II inhibitor BIRB-796 (Doramapimod) binds to p38 α with sub-nanomolar affinity.(21, 22) Yet Sullivan et al. reported nearly identical affinities of unphosphorylated and activation loop-phosphorylated forms of p38 α to BIRB-796, as well as several other type II inhibitors.(23) Therefore, for p38 α it appears that the phosphorylation status of the activation loop does not affect the conformation of the DFG motif. In this report, we further explore the relationship between activation loop phosphorylation and the conformation of the DFG motif in the non-receptor tyrosine kinases Abl and Src. To do this, we profiled a diverse panel of type II inhibitors that stabilize the DFG-out inactive form (Figure 1B) against different phospho-isoforms of these kinases. We show that the high selectivity and activation state sensitivity of imatinib is not general to all inhibitors that stabilize the DFG-out conformation, and, unexpectedly, structural features outside of the activation loop are responsible for these properties.

RESULTS AND DISCUSSION

Activation Loop-Phosphorylated and Non-Phosphorylated Src Have Similar Affinities for Type II inhibitors

We first attempted to determine whether Abl is an exception rather than the rule for the activation state-dependence of type II inhibitor potency in kinases. Since the tyrosine kinase Src is closely related to Abl,(1) we asked if this kinase would show phosphorylation state-dependent sensitivity to type II inhibitors (Figure 2A–C). To this end, we assembled a diverse panel of inhibitors that have been confirmed crystallographically to stabilize the DFG-out inactive conformation of their kinase targets. Figure 2D shows the panel of type II ligands that were used in this study: **1** (Rebastinib) and **2** (Iclusig) are potent type II inhibitors of BCR-Abl and imatinib-resistant BCR-Abl mutants,(24, 25) **4** and **5** are based on a series of type II inhibitors of Lck,(26) and the pyridinyl triazine **3** and the pyrazolopyrimidine **6** are potent inhibitors of Src.(7, 8) Importantly, all of these type II inhibitors have been demonstrated by x-ray crystallography to stabilize the DFG-out conformation in protein kinases. However, unlike imatinib, these ligands are potent inhibitors of both Abl and Src.

For biochemical studies with Src, we utilized a recombinant construct that contains a kinase domain and the regulatory SH2 and SH3 domains. To prevent any inhibitory auto-phosphorylation that could arise during our biochemical studies, the regulatory tyrosine in Src's C-terminal tail (Tyr527) (27, 28) was mutated to a non-phosphorylatable phenylalanine (Figure S1). Co-expression of this Src construct (Src Y527F) with the tyrosine phosphatase YopH in *E. coli*, followed by purification, yields homogenous kinase that is completely dephosphorylated (npY416-Src).(29) In order to generate Src that is phosphorylated at Tyr416 in the activation loop (pY416-Src), the ability of high concentrations of Src-family kinases to undergo efficient activation loop autophosphorylation was exploited.(30, 31) Quantitative activation loop phosphorylation was confirmed by immunoblot analysis and LC/MS of tryptic peptides (Figure S2).

Next, we tested the potencies of the type II inhibitors shown in Figure 2D for the npY416-Src and pY416-Src constructs in *in vitro* activity assays. In stark contrast to the reported preference of imatinib for non-phosphorylated Abl over the activation loop-phosphorylated form, most of the type II inhibitors tested demonstrated a minimal preference (< 3-fold) for npY416-Src (Figures 3A and S3). Indeed, only ligand **1** showed notable selectivity (~11-fold) between the two Src phospho-isoforms. To confirm that the observed lack of

selectivity is not due to the presence of the Y527F mutation in the C-terminal tail of npY416, we tested a subset of the type II inhibitors against unphosphorylated, wild-type (wt) Src (Table S1). As expected, the K_i s of all of the inhibitors tested are nearly the same for non-phosphorylated wt and Y527F Src. Additionally, to rule out the possibility that the lack of observed phosphorylation state-dependent inhibition is due to autophosphorylation of npY416-Src during the activity assay, we verified by immunoblot that no pY416-Src is formed under the assay conditions used (Figure S4). Finally, to determine whether the observed lack of preference is specific to Src or more general to the Src-family kinases (SFKs), we performed equivalent assays with Hck (Table S2). Similar to Src, none of the three inhibitors tested showed a > 4-fold preference for unphosphorylated Hck. Thus, it appears that the potencies of inhibitors that stabilize the DFG-out inactive conformation are not sensitive to the phosphorylation state of SFK activation loops.

To confirm that npY416-Src and pY416-Src have the same affinities for DFG-out stabilizing ligands without using an activity assay, their dissociation constants (K_d s) for a BODIPY-labeled version of inhibitor **3** were determined. This probe has been used previously to assess DFG-out conformational accessibility for a number of kinases, including Src.(32) We found the K_d of this probe for npY416- and pY416-Src to be almost identical (Figure 3B). Further, a binding dissociation experiment demonstrated that both phospho-isoforms of Src have similar binding kinetics (k_{off}) for this type II probe. This information is significant because type II inhibitors tend to have slow binding and dissociation kinetics.(22) That this rate is both slow and very similar for npY416- and pY416-Src indicates that these phospho-isoforms most likely undergo similar conformational changes in order to accommodate type II inhibitors.

Only a Subset of Type II inhibitors Prefer Non-Phosphorylated Abl

Having determined that most type II inhibitors show little preference for the unphosphorylated form of Src, we returned to Abl in order to investigate whether activation state sensitivity is specific to this kinase or to the inhibitor imatinib. First, the compounds described above were tested for their abilities to inhibit Abl. Unlike imatinib, all of the inhibitors tested have comparable potencies for nonphosphorylated Abl and Src (Figure 4A). Five other type II inhibitors were also tested against Abl: **7** (AST-487), a predicted type II inhibitor of the kinase FLT3,(33) and four analogs of **3** that have variable hinge region contacts (Figure 4B). Interestingly, inhibitors **7-11** are highly selective for Abl over Src (Figure 4C), with **7** demonstrating the highest preference (~200-fold). This observed selectivity profiles for **1-11** hold for constructs that contain just the catalytic domains of Src and Abl and for constructs that also contain the regulatory SH2 and SH3 domains of these kinases (Figure S5).

Next, the phosphorylation state-dependence of Abl inhibition by this assembled compound panel was determined using activation loop phosphorylated Abl (pY412-Abl) that was generated using a previously described procedure.(14, 15) Consistent with previous reports, imatinib and **7** are much more potent inhibitors of npY412-Abl than pY412-Abl (Figure 4D). In addition, compounds **8-11** are all > 50-fold selective for the non-phosphorylated form of Abl. In contrast, **2, 3, 5, and 6** show minimal (< 5-fold) activation state-dependent inhibition of Abl. One interesting trend that becomes apparent from analyzing these data is that inhibitors that are selective for Abl over Src show activation state-dependent inhibition of Abl, while non-selective compounds show little or no phospho-dependence. Plotting the inhibitory constants (K_i s) of the inhibitors tested for npY416-Src and pY412-Abl shows a correlation in potencies (Figure 4E). Consistent with this observation, the overall correlation in inhibitor potency is much weaker for the non-phosphorylated forms of Src and Abl (Figure S3). In essence, phosphorylated Abl behaves much like non-phosphorylated Src in

the presence of type II inhibitors. Comparison of the structures of the activation loop-phosphorylated forms of Abl and Src indicates no notable differences in their hydrophobic spines (Figure S5A). However, in contrast to the structured activation loop of Abl, a majority of Src's activation loop cannot be observed (Figure S5B). This apparent difference in activation loop order does not affect how the phosphorylated forms of these kinases interact with ligands **1–6**, but may play a role in the phospho-dependence of inhibitors **7–11**.

Based on the correlation between pY412-Abl and npY416-Src, we hypothesized that the mechanism by which certain inhibitors can discriminate between Abl activation states is linked to their selectivity for Abl over Src. Therefore, we further investigated differences in type II inhibitor sensitivity between these two kinases. Despite the overall active site similarities between Src and Abl, it has been proposed that Src pays a significant energetic penalty to adopt the DFG-out inactive conformation relative to Abl.^(34, 35) Recent molecular dynamics (MD) simulations have indicated that dissimilarities in the overall active site flexibilities of Src and Abl result in different relative stabilities of their DFG-out conformations. While our data do not provide any information on the relative dynamics of these two kinases, the fact that several of the type II inhibitors in our panel are nearly equipotent against Src and Abl indicates that Src does not likely pay a substantial energetic penalty for adopting the DFG-out inactive form compared to Abl. Furthermore, Abl-selective type II inhibitors differ from their non-selective analogs in regions that interact with the adenosine pocket and not the hydrophobic pocket created by movement of the DFG motif. Therefore, a simple energetic difference in the conformation of the DFG motif between Src and Abl is highly unlikely to account for most of the preferences of imatinib and **7–11**.

P-loop Interactions Contribute to the Potency and Abl-Selectivity of Some Type II inhibitors

Another distinctive feature of Abl's interaction with imatinib is the unique kinked conformation of its phosphate-binding loop (p-loop; also referred to as the glycine-rich loop) when bound to this drug. This conserved and highly flexible loop, which makes critical contacts with the phosphates of ATP, is located between strands $\beta 1$ and $\beta 2$ of the N-terminal lobe. The kinked orientation of Abl's p-loop allows this structural feature to form a hydrophobic cage around imatinib. A co-crystal structure of Src bound to imatinib shows that the p-loop of this kinase does not make similar contacts (Figure 5A).⁽¹⁵⁾ Indeed, MD simulations suggest that the p-loop of Src maintains an extended conformation,^(34, 35) and this loop has never been observed in a kinked conformation in crystal structures of the catalytic domain of Src.^(7, 8, 12, 15) Recent biochemical and structural studies have suggested that imatinib's interaction with Abl's flexible p-loop is a major contributor to the selectivity of this drug for Abl over Src.⁷ Furthermore, some of the most common clinically-observed imatinib-resistant mutants occur in a region of Abl's p-loop that likely disrupt van der Waals contacts and hydrophobic interactions with this drug.⁽³⁶⁾

The most notable differences between activation state-dependent and –independent inhibitors occur in regions that would be expected to project toward the p-loops of Src and Abl. Therefore, we further explored the interactions of this region with our type II inhibitor panel by determining their abilities to inhibit the catalytic activity of Abl Y253H, which is an imatinib-resistant p-loop mutant of Abl that is observed in the clinic. Mutating Tyr253 to a His residue eliminates a large hydrophobic contact between the tyrosine side chain and the pyridine and pyrimidine rings of imatinib. Consistent with previous reports, imatinib is a much less potent ($> 300\times$) inhibitor of npY412-Abl Y253H than npY412-Abl (Figure 5B). Like imatinib, the potencies of activation state-dependent inhibitors **7–11** are greatly diminished for npY412-Abl Y253H. However, activation state-independent inhibitors **1–6**

are nearly equipotent against npY412-Abl and npY412-Abl Y253H. Plotting our inhibitory constant data for pY412-Abl and npY412-Abl Y253H suggests that these two features are correlated ($R^2 = 0.82$, Figure 5C and Figure S6). Therefore, there appears to be a relationship between activation state-dependent inhibition and sensitivity to p-loop mutations.

As Abl-selective type II inhibitors (imatinib and **7–11**) are also sensitive to the phosphorylation state of the activation loop and, by corollary, p-loop mutations, these results provide further evidence that p-loop interactions contribute to selective inhibition of Abl over Src. Although co-crystal structures of all of the type II ligands in this study have not been obtained, examination of the subset of selective and non-selective type II ligand-bound structures of Src and Abl that have been determined provides at least a partial explanation for the role of Abl's p-loop. Structures of **3** and **5** bound to Abl have not been determined, but an overlay of similar type II ligands bound to other kinase targets shows that their extended aniline groups are incompatible with the closed p-loop conformation that is observed in the Abl-imatinib complex (Figure S7). While **1** does not contain a substituent that would be expected to clash with the conformation of the p-loop in the Abl-imatinib complex, inspection of the **1**-Abl complex shows that the p-loop does not make any observable contacts with this drug (Figure S8). Furthermore, the structure of another equipotent Src and Abl inhibitor, which was not characterized in this study, bound to Abl shows that this type II ligand does not form any contacts with the kinked p-loop of Abl either.(37) As described above, the p-loop of Src has not been observed in a kinked conformation, and computational studies suggest that this structural element is not able to make extensive contacts with type II inhibitors. Therefore, it appears that type II inhibitors that have similar affinities for Src and Abl either cannot or do not make extensive contacts with the p-loops of Src or Abl. Unfortunately, no crystal structures of **7–11** bound to Abl have been determined, but we predict that Abl's p-loop will make a hydrophobic cage around these inhibitors, like it does when bound to imatinib.(38)

Having demonstrated that p-loop interactions contribute substantially to the potencies of certain inhibitors for Abl, we were interested in knowing specifically how these inhibitors affect the conformation of the p-loop. Based on the divergent sensitivities of the two type II inhibitor classes to p-loop mutations, we hypothesized that **1–6** will affect the conformational preference of the p-loop of Abl differently than inhibitors **7–11** and imatinib. To probe this, a mass spectrometry-based footprinting technique was used to study the dynamics of Abl's p-loop when bound to inhibitors **2**, **3**, and **7** (Figure 6). This method uses isotope-coded affinity tagging (ICAT) reagents to ratiometrically determine the alkylation rate of cysteine residues (39) and has previously been used to study the dynamics of other protein kinases.(40, 41) Since the p-loop of Abl has no cysteines, we performed our studies using a p-loop mutant (Q252C) that has been characterized previously (Figure 6A).(42) When Abl is bound to **3**, which is sterically incompatible with a closed and kinked p-loop conformation (Figure S7), the alkylation rate of Q252C is significantly slower compared to *apo* and **7**-bound Abl (Figure 6B). Thus, these representative members of the two type II ligand classes have divergent effects on the p-loop. Next, we tested the alkylation rate of Q252C in the presence of inhibitor **2** (Figure 6C). Unlike **3**, **2** does not contain a substituent that is incompatible with a closed and kinked p-loop conformation, and a crystal structure of **2** bound to Abl shows that Tyr253 is in close proximity to the imidazopyridazine ring of this inhibitor.(43) However, **2** is similar to **3** in that it is an equipotent inhibitor of Abl and Src, and not sensitive to activation loop phosphorylation or the Y253H mutation. Consistent with these characteristics, the alkylation rates of Q252C in the Abl-**2** and Abl-**3** complexes are very similar. While mechanistically it is not entirely clear why alkylation of Q252C in Abl-**2** is slower than Abl-**7** or *apo* Abl, this behavior is consistent with the other biochemical properties of this ligand.

The relationship between the p-loop and activation loop of Abl

Knowing that both phosphorylation of Tyr412 in the activation loop and a p-loop mutation (Y253H) have similar effects on the potencies of compounds **7–11** and imatinib, we asked if there is direct communication between these two flexible loops (Figure 7A). In the imatinib-Abl complex, the p-loop is greater than 20 Å away from the activation loop residue Tyr412. However, the base of the activation loop is in close proximity to the p-loop in this complex, with Tyr253 positioned ~5 Å away from Phe382 of the DFG motif (Figure 7B). Therefore, the base of the activation loop is in reasonably close proximity to the p-loop of Abl when this flexible loop is in a kinked conformation and the interplay between these two flexible loops has previously been reported. For example, Kwarciński et. al. showed that an Abl mutant that contains a cysteine on the p-loop (Abl Q252C) reacts with an ATP-competitive irreversible inhibitor at different rates depending on the phosphorylation state of the activation loop.⁽⁴²⁾ Furthermore, we found that the rate of activation loop phosphorylation of Abl Y253H by Hck is much slower than for wt Abl (Figure S9), suggesting that mutating Tyr253 to His may alter the conformation of the activation loop.

To further investigate this relationship, we generated and tested pY412-Abl Y253H against inhibitors **2**, **3**, **7**, and imatinib. The fold K_i differences for all four inhibitors are shown in Figures 7C and 7D. As expected, compounds **2** and **3** are not significantly affected by activation loop phosphorylation of the Y253H Abl mutant. In contrast, **7** and imatinib are sensitive to the phosphorylation state of the activation loops of wt and Y253H Abl, although the fold loss in affinity for the Y253H Abl mutant appears to be less than for wt Abl. These data show that while activation loop phosphorylation may affect the conformation of the p-loop, the relationship between these two flexible active site features is complex. Biophysical studies to further probe the conformational inter-relationship between the p-loop and activation loop in Abl are currently underway.

CONCLUSIONS

In this study, we have examined how two dynamic and flexible loops in tyrosine kinases affect the potencies of inhibitors that stabilize the DFG-out conformation (type II). Based on the well-precedented observation that activation loop phosphorylation of Abl decreases its affinity for imatinib, we asked if the closely-related kinase Src would show the same effect. However, type II ligands that are equipotent against Abl and Src show very little difference in inhibition between unphosphorylated and activation loop-phosphorylated forms of Src. Our analysis was then extended to a larger group of type II inhibitors that are selective for Abl over Src. Strikingly, type II inhibitors that prefer Abl to Src are also sensitive to the phosphorylation state of the activation loop of Abl. In contrast, conformation-selective inhibitors ligands that are equipotent for Abl and Src show little phospho-dependent Abl inhibition. The fact that many inhibitors that stabilize the DFG-out conformation are not affected by activation loop phosphorylation suggests that the conformations of these two regions of the activation loop are not directly linked.

In addition, we have observed that Abl-selective type II inhibitors are highly sensitive to a clinically relevant mutation (Y253H) that disrupts a hydrophobic interaction between the p-loop and imatinib, while inhibitors that have similar affinities for Src and Abl are not. These results provide further evidence that imatinib's interaction with the closed and kinked p-loop of Abl makes a major contribution to the observed selectivity of this drug. While co-crystal structures of the other Abl-selective type II inhibitors that were used in this study (**7–11**) are currently not available, it is likely that these compounds make similar interactions with the p-loop of Abl. Type II inhibitors that do not discriminate between Src and Abl either cannot or do not make extensive interactions with this flexible loop. For type II inhibitors of Abl, there is also a correlation between sensitivity to p-loop mutations and activation loop

phosphorylation. The base of Abl's activation loop is in reasonable proximity to the closed and kinked p-loop of imatinib-bound Abl, and it is possible that there is direct energetic coupling between these two flexible loops. However, the mechanism by which the conformation of these two loops is communicated is not entirely clear, and it cannot be ruled out that the observed correlation is entirely coincidental. Currently, we are performing biophysical and biochemical analyses to elucidate the allosteric network between the activation and p-loop of Abl. Furthermore, the generality of our observations is being explored in other kinases.

EXPERIMENTAL PROCEDURES

Recombinant proteins were expressed and purified as described.(29, 44) Fluorescence measurements were performed as described.(32, 45) Abl was activated as described (15) except for Abl Y253H, which required special conditions that are noted the Supplementary Text.

Cloning and mutagenesis

Bacterial expression plasmids containing genes encoding Src 3D, Src KD, Hck 3D, and Abl KD were kindly provided by J. Kuriyan. All mutagenesis was performed by Quikchange (Agilent).

Synthetic methods

Imatinib was purchased from ChemieTek. **1**,(46) **2**,(24) **3**,(15) **4**,(41) **6**,(7) **7**,(33) and **11** (47) were made as described. Synthetic methods for **5** and **8–10** are described in the Supplementary Text.

Activation of Src

Src Y527F (250 nM) was incubated in buffer (50 mM MOPS [pH 7.4], 67 mM NaCl, 10 mM MgCl₂, 0.001% (v/v) Tween 20) with ATP (1 mM) at 25 °C or 37 °C for 3 h. Quantitative phosphorylation was determined by immunoblot using antibodies specific for phospho- and non-phospho-Src Tyr416 (Cell Signaling) and LC/MS of tryptic digests (performed as described (41)).

Activity assays

These assays were performed as described.(8, 15, 48) In cases where cold ATP was used to activate the kinase prior to the assay, the activation mixture was diluted sufficiently to contribute at most 2 μM to the final reaction, and thus IC₅₀ ≈ K_i.

ICAT footprinting

Labeling reagents were made as described,(39) and experiments were performed generally as described.(40) Briefly, one 3-μM stock of protein in 50 mM Tris (pH 8.0), 50 mM KCl, 5 mM MgCl₂, and 0.5 TCEP-HCl was divided into aliquots, and inhibitor (10 μM) was added to yield final solutions containing 1% (v/v) DMSO. Heavy labeling reagent was added to the protein solutions, and aliquots were taken at specified times and quenched with excess DTT. Samples were precipitated with 0.02% (w/v) sodium deoxycholate and 10% (w/v) trichloroacetic acid on ice for 30 min. The precipitated protein was pelleted and washed with cold acetone, then resuspended in 8 M urea with light labeling reagent. After incubation in the dark for 30 min, the solutions were diluted with 210 μL Tris pH 8.0, 5.7 mM CaCl₂, and 1 μg porcine trypsin (Sigma), and incubated at 37 °C overnight. Samples were analyzed on a Thermo Finnigan LTQ mass spectrometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (R01GM086858 and R21CA177402) (D.J.M.), Alfred P. Sloan and Camille and Henry Dreyfus Foundations (D.J.M.), and a predoctoral fellowship from the American Heart Association (S.B.H.).

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Figure 1.

Specific ATP-binding site conformations that have been observed in Src and Abl. a) The active conformation of Abl (from the Abl-dasatinib complex (PDB ID: 2GQG)). The catalytic (orange) and regulatory (blue, yellow, and magenta) spines are shown in surface form. Helix α C is shown in yellow, and the activation loop in magenta. The catalytic glutamate residue in helix α C (Glu286) is shown in stick form. b) Abl in the DFG-out inactive conformation (from the imatinib-Abl complex (PDB ID: 1IEP)). The movement of the DFG motif phenylalanine (magenta, stick form) causes a disruption in the regulatory spine. c) Abl in the CDK-like inactive conformation (from a bisubstrate inhibitor-Abl complex (PDB ID: 2G1T)). In this inactive form, catalytic Glu286 (yellow, stick form) is rotated out of the active site, and the catalytic spine is disconnected from the regulatory spine.

Figure 2.

The ATP-binding site of Src. a) Crystal structure of Src in the active conformation (bound to an ATP analogue [PDB ID: 2SRC]). b) The DFG-out inactive conformation (Figure 1B) of Src (bound to a type II inhibitor [PDB ID: 3G6G]). c) A close-up view of the activation loop of Src. Tyr416 (purple) is located ten residues C-terminal to the DFG motif, which is shown in green for all structures. d) Type II inhibitors known to stabilize the DFG-out inactive conformation of tyrosine kinases.

Figure 3. pY416- and npY416-Src have similar potencies for type II inhibitors **1-6**. a) Fold-difference in K_i s of **1-6**. With the exception of **1**, the K_i s between the two phospho-isoforms of Src is three-fold or less. b) Direct binding (top) and dissociation (bottom) of pY416-Src (green) and npY416-Src (black) to a BODIPY-conjugated derivative of **3**.

Figure 4.

pY412-Abl and npY416-Src have similar sensitivities to type II inhibitors. a) K_i s of inhibitors **1-6** for npY412-Abl and npY416-Src. b) The structures of Abl-selective type II inhibitors **7-11**. c) K_i s of inhibitors **7-11** against npY412-Abl and npY416-Src. d) Comparison of the K_i s of **1-11** and imatinib for phosphorylated and unphosphorylated forms of Abl and Src. The asterisk indicates that the K_i for imatinib for Src could not be determined. e) A plot of the K_i s of inhibitors **1-11** for pY412-Abl and npY416-Src. The K_i s of **1-11** for pY412-Abl are plotted on the y-axis, while the K_i s of **1-11** for npY416-Src are plotted on the x-axis.

Figure 5.

Activation state-dependent inhibitors of Abl also rely on the p-loop for potency. a) Superimposition of Abl-imatinib (salmon, inhibitor in yellow) (PDB ID: 1IEP) with Src-imatinib (teal, inhibitor in white) (PDB ID: 2OIQ). The p-loop of Abl assumes a closed and kinked conformation, whereas that of Src is extended. b) Fold differences of inhibitors between Abl wild-type and Y253H demonstrate that only ligands 7–11 and imatinib appear to make extensive interactions with the p-loop. c) Comparison of K_d s for npY412-Abl Y253H and pY412-Abl.

Figure 6.

Type II inhibitors differentially alter the alkylation rate of a cysteine residue in the p-loop. a) Crystal structure (PDB ID: 1IEP) of Abl in complex with imatinib (shown in white). Gln252 is shown in orange. b) ICAT-based alkylation timecourse of Cys252 Abl alone (black) or bound to **3** (red) or **7** (blue). c) Same experiment as described in B, but with **2** (green).

Figure 7. Phosphorylation state-selectivity and sensitivity to p-loop mutations. a) A model for the hypothesis that activation loop phosphorylation disrupts the conformation of the p-loop, which in turn abrogates ligand inhibition. The p-loop is shown in red, and the activation-loop is shown in cyan. b) Crystal structure of Abl (PDB ID: 1IEP) with the distance between Tyr253 and Phe382 measured to be ~5 Å. The p-loop is shown in orange, and the activation loop is shown in cyan. c) Fold differences between pY412-Abl, npY412-Abl Y253H, pY412-Abl Y253H. d) Fold differences between phosphorylated and unphosphorylated forms of Abl and Abl Y253H. The asterisks in C and D indicate that no inhibition was observed for pY412-Abl Y253H against imatinib up to 5000 nM (Although the K_i is higher, 5000 nM was used to calculate fold differences).