Equally Potent Inhibition of c-Src and Abl by Compounds that Recognize Inactive Kinase Conformations

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Abstract

Imatinib is an inhibitor of the Abl tyrosine kinase domain that is effective in the treatment of chronic myelogenenic leukemia. Although imatinib binds tightly to the Abl kinase domain, its affinity for the closely related kinase domain of c-Src is at least 2,000-fold lower. Imatinib recognition requires a specific inactive conformation of the kinase domain, in which a conserved Asp-Phe-Gly (DFG) motif is flipped with respect to the active conformation. The inability of c-Src to readily adopt this flipped DFG conformation was thought to underlie the selectivity of imatinib for Abl over c-Src. Here, we present a series of inhibitors (DSA compounds) that are based on the core scaffold of imatinib but which bind with equally high potency to c-Src and Abl. The DSA compounds bind to c-Src in the DFG-flipped conformation, as confirmed by crystal structures and kinetic analysis. The origin of the high affinity of these compounds for c-Src is suggested by the fact that they also inhibit clinically relevant Abl variants bearing mutations in a structural element, the P-loop, that normally interacts with the phosphate groups of ATP but is folded over a substructure of imatinib in Abl. Importantly, several of the DSA compounds block the growth of Ba/F3 cells harboring imatinib-resistant BCR-ABL mutants, including the Thr315Ile “gatekeeper” mutation, but do not suppress the growth of parental Ba/F3 cells. Cancer Res 2009;69(6):2384–92

Introduction

The kinase inhibitor imatinib (Gleevec, Novartis) has enabled highly effective therapy for chronic myelogenic leukemia (CML; refs. 1, 2). The clinical success of imatinib is due partly to its high affinity for the tyrosine kinases BCR-ABL, c-Abl, c-Kit, and platelet-derived growth factor receptor (PDGFR) combined with much reduced affinity for most other kinase targets, including the Src family kinases that are most closely related to Abl. Simple comparisons of sequence and structure do not provide an explanation for why imatinib is inactive against c-Src. The structure of the c-Src-imatinib complex closely resembles that of the Abl-imatinib complex, despite the 2,300-fold difference in affinity between c-Src and Abl kinase domains for imatinib (3).

Imatinib is derived from a 2-phenylaminopyrimidine scaffold (Fig. 1B, rings C and D) that is substituted with a pyridine (ring B) and a benzamide-piperazine (rings E and F). Imatinib is bound deeply in the cleft between the N-lobe and C-lobe of the kinase via three binding sites (Fig. 1A). The pyridine and pyrimidine moieties (rings B and C) bind to the site normally occupied by the adenine group of ATP (Fig. 1A, adenine pocket). The meta-diaminophenyl and benzamide group (rings D and E) are accommodated within a pocket that is enlarged greatly by adoption of an inactive conformation by the kinase domain (Fig. 1A, specificity pocket). The methylpiperazine group (ring F) binds to a hydrophobic patch on the surface of the kinase domain (Fig. 1A, exposed site). Imatinib makes only four hydrogen bonds with Abl, and the majority of interactions are mediated through van der Waals contacts, with the burial of ∼1,300 Å² of surface area between the drug and the protein (4).

The adoption of a specific inactive conformation by the kinase domain of Abl is key to the recognition of imatinib (5). Three distinct conformations of the kinase domains of c-Src and Abl have been described structurally: an active conformation (Fig. 2, state A); a c-Src/Cdk–like inactive conformation (Fig. 2, state B), which is similar to the inactive conformations of Src family kinases and cyclin-dependent kinases (Cdk; refs. 6–8); and an Abl/c-Kit–like inactive conformation (Fig. 2, state C, refs. 5, 9, 10), which was first observed in the imatinib complexes of those kinases. The conformation of the conserved Asp-Phe-Gly (DFG) motif at the beginning of the activation loop is a key feature that distinguishes between these conformations. In the active conformation (Fig. 2, state A), the aspartate side chain faces into the ATP binding pocket and coordinates a Mg2+ ion, and the phenylalanine side chain is rotated out of the ATP binding pocket (DFG Asp-in). In the Abl/c-Kit–like inactive conformation (Fig. 2, state C), the DFG motif is flipped, the phenylalanine side chain occupies the ATP binding pocket, and the aspartate side chain faces away from the active site (DFG Asp-out). In the c-Src/Cdk–like inactive conformation (Fig. 2, state B) the DFG conformation is intermediate between DFG Asp-out and DFG Asp-in.

It has been assumed that the low affinity of imatinib for c-Src is a consequence of an inability of c-Src to adopt the Abl/c-Kit-like inactive conformation. That the explanation is not so simple is indicated by the crystal structures of imatinib bound to c-Src (3) and theSrc-family kinase Lck (11). These crystal structures showed that when c-Src and Lck bind to imatinib they adopt the Abl/c-Kit–like inactive conformation, with the DFG motif flipped into the DFG Asp-out conformation.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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©2009 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-08-3953

Published OnlineFirst March 10, 2009; DOI: 10.1158/0008-5472.CAN-08-3953
The difference in imatinib affinity for c-Src and Abl cannot be explained by the loss of hydrogen bonds or by steric occlusion. In terms of contacts with the drug, the differences between the c-Src and Abl complexes are limited to a strand-loop-strand motif called the P-loop, which interacts with the phosphate groups and the ribose group of ATP in active kinases. In the Abl-imatinib complex, the P-loop is folded over the pyridine and pyrimidine groups (Fig. 1B, rings B and C) of imatinib, shielding them from water molecules. This conformation was unclear for the following reasons: (a) the P-loop conformation is in a hydrophobic face of the pyridine ring (Fig. 1B, ring B) the P-loop.

Figure 1. Structure and binding orientation of inhibitors that recognize the Abl/c-Kit–like inactive conformation of protein kinases. A, schematic representation of the subsites that are occupied by inhibitors that bind the DFG Asp-out conformation of Abl. Red, P-loop; orange, helix αC; blue, activation loop. The three distinct subregions that are occupied by imatinib (adenine pocket, specificity pocket, and exposed site) are represented with dotted circles. The letters used to label imatinib in this model directly correlate to the structure in B. B, chemical structures of imatinib, DSA1, and DSA8. All molecules are drawn in an orientation that is consistent with their mode of binding to c-Src or Abl. Red, functional groups, which are similar between the inhibitors. Each ring in these structures is labeled (rings A–F) for reference.

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P-loop that Abl adopts when bound to imatinib. In contrast, the P-loop is undistorted in the c-Src-imatinib complex (Fig. 3).

The functional relevance of these differences in the P-loop conformation was unclear for the following reasons: (a) the P-loop is extended in the high-affinity c-Kit-imatinib complex (9), as it is in c-Src (3); (b) the P-loop seems to make nonspecific hydrophobic contacts with imatinib; and (c) replacement of the P-loop in c-Src by the corresponding residues in Abl did not increase the affinity for imatinib (3). Also, Lck has a very similar P-loop sequence but has a higher affinity for imatinib than does c-Src (3). For these reasons, we have speculated previously that the Abl/c-Kit–like conformation may be associated with a relatively high free energy in c-Src and that the low affinity of imatinib for c-Src might result from the energy required to convert the c-Src/Cdk–like inactive conformation to the Abl/c-Kit–like inactive conformation (3).

About 35% of all CML patients undergoing imatinib treatment accumulate mutations in the Abl kinase domain that render the kinase resistant to imatinib (12, 15). The energetic balance between different conformations of the kinase domain is relevant for understanding the mechanisms by which mutations in the kinase domain of Abl reduce imatinib affinity. Even in cases wherein mutations result in steric blockage of imatinib binding, this conformational balance may be relevant. For example, a mutation (Thr315Ile) at the gatekeeper position of Abl (so named because of its location at the junction of the adenine pocket and the specificity pocket) leads to the activation of the mutant protein in vivo (16). This gatekeeper mutation is of particular clinical interest because it results in resistance to second generation Abl kinase inhibitors, such as dasatinib and nilotinib (17–19). In addition, mutation at the equivalent position in c-Kit and PDGFR kinase causes resistance to imatinib (20, 21). Mutation at the gatekeeper residue in epidermal growth factor receptor kinase causes clinical resistance to gefitinib and erlotinib by increasing the affinity for ATP (22, 23).

In this study, we have generated a series of inhibitors (DSA1–DSA9) that are based on the central chemical scaffold of imatinib (Fig. 1B) and that are designed to bind to kinases with a flipped DFG conformation. We find that these derivatives, unlike imatinib, are equipotent inhibitors of both c-Src and Abl, with inhibitory constants in the nanomolar range. Crystal structures of the c-Src kinase domain bound to two of these inhibitors (DSA1 and DSA8) reveal that c-Src readily adopts the DFG Asp-out conformation, which is supported by kinetic data.

Clearly, DFG flips are not hindered in the c-Src kinase domain. Instead, our results indicate that the different accommodations of the hydrophobic face of the pyridine ring (Fig. 1B, ring B) of imatinib by the P-loop of c-Src and Abl are the key to understanding the selectivity of this drug (Fig. 3).
Materials and Methods

Protein purification and kinetic assays. All kinases were expressed and purified, as previously described (24). Stopped-flow kinetics of drug binding was performed, as previously described (25).

Crystallization and structure determination. Crystals of the Src-DSA complexes grew in hanging drops (1 μL of protein + 1 μL of mother liquor) at 20°C overnight (Supplementary Table S1). Crystals were cryoprotected in mother liquor plus 20% glycerol, frozen, and stored in liquid nitrogen. The structures were solved by molecular replacement in Phaser (26), built in Coot (27), and refined with REFMAC5.4 (28).

Cell growth viability studies of Ba/F3 cells harboring BCR-ABL kinase domain mutations. Exponentially growing Ba/F3 cells (1 × 10⁵) were plated in media containing interleukin-3 (IL-3; parental Ba/F3 cells) or lacking IL-3 (BCR-ABL–expressing Ba/F3 cells) or lacking IL-3 (BCR-ABL–expressing Ba/F3 cells) in the presence of varying concentrations of DSA7 or DSA8 for 48 h. The number of trypan blue–excluding cells were determined using an automated viable cell counter, as previously described, and normalized to the mock-treated sample (29). Experiments were performed in triplicate. BCR-ABL amino acid substitutions were numbered according to the Abl type 1a convention.

Results and Discussion

Equipotent inhibitors of the tyrosine kinases c-Src and Abl that are designed to recognize flipped DFG motifs. We were intrigued by a recent report describing a series of inhibitors that bind to the DFG Asp-out conformation of the receptor tyrosine kinase Tie-2 (30). Lead compounds from this inhibitor series were reported to be potent inhibitors of the kinases p38α, c-Kit, Lck, Lyn, and c-Src (IC₅₀ ≤25 nmol/L; ref. 30). We were interested in determining the conformation that c-Src adopts when bound to inhibitors based on these scaffolds. To this end, we synthesized pyridinyl triazine DSA1 (Fig. 1B; Table 1). DSA1 contains many of the same functional groups that are exploited by imatinib to bind to the DFG Asp-out conformation of Abl. The exocyclic nitrogen from the trimethoxaniline ring (ring A) and one of the nitrogens from the triazine ring (ring B) are expected to form hydrogen bonds with the hinge region in an analogous manner to the pyridyl nitrogen (ring B) from imatinib. In addition, the amide connecting the 3-trifluoromethylphenyl group (ring E) and the meta-diamino phenyl (ring D) are expected to form key hydrogen bonds with a glutamate residue in the α-C helix and a backbone amide in the DFG motif. Finally, the 3-trifluoromethylphenyl group (ring E) is expected to occupy the specificity pocket (Fig. 1A).

In stark contrast to imatinib, DSA1 is a nearly equipotent inhibitor of c-Src (IC₅₀, 4.6 nmol/L), the Src family kinase Hck (IC₅₀, 10 nmol/L), and Abl (IC₅₀, 10 nmol/L) in an in vitro kinase assay (Table 1). A small panel of triazine derivatives was generated (Table 1, DSA2–DSA9). Variation of the functionality around the pyridinyl triazine core had a small effect on the potency and selectivity of these compounds for Abl, c-Src, and Hck, and all of the compounds from this series are equipotent inhibitors of these kinases. Reversing the orientation of the amide that connects the 3-trifluoromethylbenzamide (ring E) and the metadiaminophenyl ring (ring D; DSA2) causes a small decrease in potency for all three kinases but no change in selectivity. Replacing the linkage between these two moieties with a urea group (DSA3) increases the potency for Src, Hck, and Abl but does not alter selectivity. Substituting the meta-diaminophenyl with a meta-aminophenol (DSA6) has little effect on potency or selectivity. Finally, the nature of the functionality that is believed to occupy the specificity pocket seems to have a small effect on the potency and selectivity of these compounds. Clearly, the equipotent inhibition of Abl and c-Src shown by the DSA compounds is not due primarily to compensating binding interactions in the specificity pocket.

Structure of c-Src kinase domain bound to DSA8. The general binding mode of DSA8 in the c-Src-DSA8 complex is similar to that of...
imatinib in the c-Src-imatinib complex (Fig. 4B). DSA8 forms a total of four hydrogen bonds with the c-Src kinase domain, and a total of 1,294 Å² surface area is buried between the drug and the protein.

Rings D-F are chemically identical between imatinib and DSA8, and their interactions with c-Src are essentially the same (Fig. 4). Similar to the imatinib complex, the meta-diaminophenyl and benzamide groups (rings D and E) of DSA8 dock into the specificity pocket. The amide carbonyl group that connects rings D and E hydrogen bonds with the backbone of Asp404 in the DFG motif and the adjacent amino group hydrogen bonds to the side chain of Glu310 in helix αC (Fig. 4). As for imatinib, the methylpiperazine (ring F) of DSA8 binds to the exposed site.

In DSA8, the triazine and pyridine groups (rings B and C) occupy the adenine binding pocket that imatinib occupies with pyridine and pyrimidine groups (rings B and C) but form different interactions. The pyridine in DSA8 is ortho-substituted, whereas the equivalent pyrimidine in imatinib is meta-substituted. This leads to a very different accommodation of the pyridine and triazine groups of DSA8 relative to the pyrimidine and pyridine groups in imatinib. The pyridine group of DSA8 fits more deeply into the hydrophobic groove within the adenine pocket formed by the side chains of Val281 and Phe405 than the pyrimidine of imatinib.

Inhibitor binding kinetics indicate that DFG flips are involved in DSA binding. The crystal structures of the complexes of DSA1 and DSA8 show that the c-Src kinase domain can bind to these inhibitors in the DFG-flipped conformation. One concern arises from the fact that both complexes are in the same crystal form as the c-Src-imatinib complex, raising the possibility that the DFG-flipped conformation is influenced by crystal packing interactions. This is unlikely to be the case, because this crystal lattice is consistent with both active and inactive conformations of c-Src (3). Nevertheless, we sought additional support for the importance of the flipped DFG conformation in the binding of DSA1 and DSA8.

We have developed an assay to monitor the kinetics of inhibitor binding to Abl and Src kinases (3). By choosing conditions under which binding follows pseudo-first-order kinetics, we can determine the rate constants for binding ($k_{on}$) and for dissociation ($k_{off}$; ref. 31). For a two-state reaction, the dependence of the observed rate constant on inhibitor concentration is linear, and any deviation from linearity indicates additional rate-limiting steps. We have shown that, for Abl, the rate-limiting step at high inhibitor concentrations is the DFG flip rather than the rate of encounter between the inhibitor and the kinase (25). The observed rate constant for imatinib binding to Abl depends nonlinearly on inhibitor concentration, and the binding rate constant decreases with increasing pH. We have shown, using mutagenesis, that this pH dependence is due to deprotonation of the aspartate side chain in the DFG motif, which is expected to thermodynamically disfavor the DFG Asp-out conformation and to effectively slow down the DFG flip (25).

We monitored the binding kinetics of DSA1 and DSA8 to the kinase domains of c-Src and Abl (Supplementary Fig. S1). We found...
that the observed rate constants for binding do not increase linearly with inhibitor concentration, consistent with the encounter between the inhibitor and kinase not being rate limiting at high inhibitor concentration. We monitored the binding kinetics at pH 8.0 and pH 5.8 and found that, for both c-Src and Abl, the binding rate constants decrease with increasing pH, consistent with the DFG flip being rate limiting, as shown previously for the Abl/imatinib complex (25).

One difference between the binding of imatinib and the DSA compounds to Abl is the 10-fold lower maximal observed rate constant for the DSA compounds (Supplementary Fig. S1; ref. 3). This difference may be due to the conformation of the P-loop in the imatinib complex, where it is kinked, and the DSA complexes, where it is extended. We have no experimental information concerning the preferred conformation of the P-loop of Abl in the absence of the inhibitors. One interpretation of our data is that the P-loop of Abl is more stable in the kinked conformation and that it is in the extended conformation, required for DSA binding, only 10% of the time.

**DSA compounds are potent inhibitors of the gatekeeper threonine mutation in c-Src and Abl.** The DSA series of inhibitors show *in vitro* activity against the gatekeeper mutation.
Figure 4. Structures of wt c-Src imatinib (A, PDB entry 2OIQ; ref. 3), wt c-Src DSA8 (B, PDB entry 3G6G), and Thr338lle c-Src DSA1 (C, PDB entry 3G6H). In all three complexes, c-Src is in an Abl/c-Kit–like inactive conformation with a flipped DFG motif. Top, structure of the active site region of these complexes. c-Src is shown in gray ribbons. Red, P-loop; orange, helix αC; blue, DFG motif. Parts of the protein that obscure the view of the inhibitors have been removed for clarity. Each ring in DSA1, DSA8, and imatinib has been labeled according to Fig. 1B. Bottom, schematic diagram of the binding interactions made by DSA1, DSA8, and imatinib with c-Src. Red semicircles, hydrophobic contacts; green dotted lines, H bonds with their respective lengths. The yellow arrow in the depiction of DSA1 and DSA8 indicates the rotation of ring D relative to imatinib. Schematic drawings were prepared with LigPlot (34). D, proliferation of parental BaF3 cells in the presence of IL-3 and Ba/F3 BCR-ABL cells in the absence of IL-3 in response to a dose titration of DSA8. Error bars, SEM.
in both Abl and c-Src kinase domains (Table 2) with IC50 values in the nanomolar range. The gatekeeper residue in c-Src is Thr338. DSA1 to DSA9 inhibit c-Src Thr338Ile with only a 2-fold to 8-fold increase in IC50 values over that for wild-type (wt) c-Src (Table 2). These compounds also inhibit Abl Thr315Ile with only a 2-fold to 20-fold increase in IC50 with the two of the most common mutations in the P-loop (Tyr253His and Glu255Val), which together account for ~30% of the clinically observed resistance mutations (14). The corresponding mutants were also generated in c-Src (Phe278His and Glu280Val). These mutations are likely to disrupt the kinked conformation of the P-loop that is seen in the Abl-imatinib complex (12). The side chain of Tyr253 stabilizes the P-loop distortion through a hydrogen bond with the backbone and side chain of Asn322 in the C-lobe, and it engages in a perpendicular aromatic-aromatic interaction with the phenylalanine side chain of the DFG motif. The smaller histidine side chain cannot engage in either of these interactions, and the kinked conformation is likely to be destabilized. The Tyr253His mutant is inhibited in vitro with nanomolar affinity by all the DSA compounds, and their potency against this mutant is 10-fold to 100-fold higher than imatinib (Table 2).

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NOTE: All activity assays were performed in triplicate or quadruplicate. Abbreviation: N/D, not determined.

*Full-length Abl Thr315Ile was obtained from a commercial source (Invitrogen), whereas the other proteins are kinase domain constructs expressed and purified from bacteria.

The crystal structure of DSA1 bound to c-Src Thr338Ile closely resembles that of DSA8 bound to wt c-Src (Fig. 4C). A total of 1214 Å2 of solvent accessible surface area is buried between protein and DSA1, which lacks the interactions provided by the piperazine group (ring F) in imatinib and DSA8. Instead, it is anchored by the trifluoromethyl into a hydrophobic pocket composed of the side chains of Val313, Leu317, Leu322, Val377, Tyr382, and Val402 (Fig. 4C).

The reason for the observed gain in activity toward the gatekeeper mutant seems to be due to the rotation of the meta-diaminophenyl (ring D) in DSA1 relative to imatinib (Fig. 4). This leads to the displacement of the linker amine group between rings C and D in DSA1 by 1 Å relative to its position in the c-Src-imatinib or Abl-imatinib complexes. Whereas the displacement of the amine linker in DSA1 breaks the hydrogen bond between the side chain hydroxyl of Thr338 and the linker amine of imatinib, it also prevents the steric clashes that would otherwise occur due to the bulkier isoleucine side chain in the Thr338Ile gatekeeper mutation. However, the loss of the hydrogen bond to Thr338 is compensated by a hydrogen bond between the amine preceding the trimethoxybenzene (ring A) of DSA1 and the backbone carbonyl of Met341.

**DSA compounds are active against some Abl variants with mutations in the P-loop.** Resistance mutations localize most frequently to the P-loop of the Abl kinase domain. We tested the potency of three DSA compounds against Abl kinase domains with the two of the most common mutations in the P-loop (Tyr253His and Glu255Val), which together account for ~30% of the clinically observed resistance mutations (14). The corresponding mutants were also generated in c-Src (Phe278His and Glu280Val). These mutations are likely to disrupt the kinked conformation of the P-loop that is seen in the Abl-imatinib complex (12). The side chain of Tyr253 stabilizes the P-loop distortion through a hydrogen bond with the backbone and side chain of Asn322 in the C-lobe, and it engages in a perpendicular aromatic-association interaction with the phenylalanine side chain of the DFG motif. The smaller histidine side chain cannot engage in either of these interactions, and the kinked conformation is likely to be destabilized. The Tyr253His mutant is inhibited in vitro with nanomolar affinity by all the DSA compounds, and their potency against this mutant is 10-fold to 100-fold higher than imatinib (Table 2).

Glu255Val is the most common imatinib-resistant mutation located in the P-loop of Abl. The Glu255 side chain engages in a hydrogen-bonding network with Lys247 and Tyr257 that stabilizes the antiparallel β-strand of the P-loop just before it kinks toward the C-lobe of the kinase domain. The DSA compounds tested show a 2-fold to 20-fold higher in vitro potency against this mutation than does imatinib (Table 2). Not only are DSA compounds potent against P-loop mutants of Abl, their loss in affinity for these mutants relative to wt Abl is much less than that of imatinib. We tested the effect of mutations in the c-Src kinase domain on DSA binding (Table 2). Strikingly, the P-loop mutations do not affect the binding of c-Src to the DSA compounds.

The DSA compounds are also active against other resistance mutations, e.g., Phe359Val, which is located in the exposed site (Fig. 1A). This mutation was screened against three DSA compounds to test the effect of substitutions on ring E (piperazine versus trifluoromethyl). We find that the DSA compounds are potent inhibitors of the Abl Phe359Val mutation independent of the substitution on ring E (Table 2). Likewise, the DSA compounds tested inhibit wt c-Src and c-Src Tyr382Val with similar potency (Table 2).
Conclusions

In this study, we present a series of inhibitors with two important features. They equipotentially inhibit the c-Src and Abl kinase domains in the inactive DFG Asp-out conformation with nanomolar affinity. They are also effective against resistance mutations in Abl, including the Thr315Ile mutation at the gatekeeper position and mutations in the P-loop. The sensitivity of the P-loop mutants to the DSA compounds points to the importance of interactions at the adenine pocket rather than the DFG motif and the specificity site, as being critical for the ability to distinguish between Abl and Src. It seems that the tight binding of Abl requires a high level of solvent exclusion at the adenine pocket. In the Abl-imatinib complex, this is provided by the kinked conformation of the P-loop, which effectively closes off a hydrophobic cage for the B and C rings of imatinib (Fig. 3; ref. 5). In the c-Kit-imatinib complex, the P-loop is in an extended conformation, but an insertion in the activation loop (absent in Abl and c-Src) serves to close off the adenine pocket when imatinib is bound (9). The DSA compounds, which engage the adenine pocket with more polar groups, do not seem to require the formation of this hydrophobic cage.

The key to imatinib selectivity also seems to be its Achilles heel: high-affinity imatinib binding to Abl requires solvent shielding through a kinked P-loop conformation, which can be disrupted by resistance mutations. This study shows that both c-Src and Abl can readily access the DFG Asp-out conformation and that it is possible to target the clinically important gatekeeper mutation by inhibitors that are specific for the DFG Asp-out conformation. Furthermore, the DSA compounds may prove to be valuable leads for the development of new therapeutics for the treatment of drug-resistant CML as shown by their ability to potently and selectively block the proliferation of Ba/F3 cells expressing clinically relevant BCR-Abl mutants while sparing parental Ba/F3 cells. Notably, the DSA compounds are able to block the growth of cells expressing the Thr315Ile mutation, which poses an increasingly significant clinical problem.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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