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Conformation-selective analogs of dasatinib reveal insight into kinase inhibitor binding and selectivity

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Abstract

In the kinase field, there are many widely held tenants about conformation-selective inhibitors that have yet to be validated using controlled experiments. We have designed, synthesized, and characterized a series of kinase inhibitor analogs of dasatinib, an FDA-approved kinase inhibitor that binds the active conformation. This inhibitor series includes two Type II inhibitors that bind the DFG-out inactive conformation and two inhibitors that bind the α C-helix out inactive conformation. Using this series of compounds, we analyze the impact that conformation-selective inhibitors have on target binding and kinome-wide selectivity.

INTRODUCTION

Protein kinases (PKs) play a key role in cellular signal transduction pathways and regulate important cellular processes such as cell growth through post-translational phosphorylation. PKs are one of the largest protein families; the human kinome encodes for 518 protein kinases, which corresponds to 1.7% of the entire genome.¹ For these reasons, they continue to be an attractive target for drug discovery.

Structural analysis of the FDA-approved kinase inhibitors reveals three distinct kinase conformations amenable to inhibitor binding: DFG-in active, DFG-out inactive, and α C-helix out inactive.^{2–5} The majority of approved kinase inhibitors (19 of 30) bind the active kinase conformation and these inhibitors have previously been classified as ‘Type I’

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ACCESSION NUMBERS

Crystallographic coordinates for the **DAS-DFGO-I:c-Src**, **DAS-CHO-I:c-Src** and **DAS-CHO-I:c-Abl** complexes have been deposited at the RCSB Protein Data Bank under accession numbers 4YBJ, 4YBK, 4YC8 (respectively).

SUPPLEMENTAL DATA

Supplemental Data include five figures and three tables and can be found with this article online.

inhibitors. There are 7 approved kinase inhibitors that bind the inactive, DFG-out conformation and these inhibitors have been termed 'Type II' inhibitors. Finally, 4 approved inhibitors bind an alternate inactive conformation, the α C-helix out conformation.

Type I inhibitors are the most common kinase inhibitor class, both among approved and non-approved inhibitors. These inhibitors bind their target kinase(s) in a manner similar to ATP, where the kinase is in an active conformation (in which the activation loop is poised for phosphate transfer).⁶ Meanwhile, Type II inhibitors bind an inactive kinase conformation in which the activation loop is forced to undergo a large conformational change due to the kinase inhibitor occupying an allosteric pocket adjacent to the nucleotide-binding site.⁷ The specific inactive conformation bound by Type II inhibitors is termed the DFG-out inactive conformation. In this conformation, the conserved Asp-Phe-Gly (DFG) motif required for catalysis is flipped outward so that the Asp faces into the solvent and can no longer coordinate ATP/Mg²⁺. Finally, α C-helix out inhibitors stabilize the kinase in a conformation where a key salt bridge between the catalytic lysine and a glutamate in helix α C is disrupted due to displacement (and/or outward rotation) of the α C-helix.^{8–12}

Because the kinase ATP pocket is highly conserved, developing selective kinase inhibitors remains a key challenge for the drug discovery and chemical biology fields. Since the discovery of imatinib,^{13,14} a selective Type II inhibitor of c-Abl, the prevailing opinion has been that Type II inhibitors are more selective than their Type I counterparts.^{15,16} It is commonly believed that not all kinases can adopt the DFG-out conformation.^{17–19} These beliefs originate in the finding that imatinib does not inhibit c-Src, a protein kinase with homology to c-Abl.^{20,21} The prevailing hypothesis was that imatinib did not bind c-Src, because c-Src cannot adopt the DFG-out conformation.²² However, it was later shown that c-Src can in fact readily adopt the DFG-out conformation.^{23–26} Kinome-wide analyses of kinase inhibitors initially strengthened the idea that Type II inhibitors were more selective,²⁷ however, recent analyses suggest that the DFG-out conformation is conserved across the kinome.^{7,28} Nonetheless, Type II inhibitors are still today claimed to be more selective than Type I kinase inhibitors.²⁹ No comprehensive selectivity analysis of inhibitors that bind the α C-helix out (CHO) conformation has been performed, however, three of the FDA-approved inhibitors that bind this specific inactive conformation (lapatinib, vemurafenib, and ibrutinib) are among the most selective kinase inhibitors known.^{27,30,31} Complicating matters, CHO inhibitors are often incorrectly considered Type I inhibitors in analyses of kinome profiling data.^{7,18,32}

In an effort to understand the role of conformation on the selectivity of kinase inhibitors, we have created a set of conformation-selective analogs of dasatinib, an FDA-approved pan-kinase inhibitor.⁴ Dasatinib is a potent inhibitor of c-Src (along with many other tyrosine kinases) and binds the active kinase conformation.^{33,34} Herein, we designed analogs of dasatinib that bind the DFG-out and α C-helix out conformations of c-Src. Crystallographic characterization of our conformation-selective dasatinib analogs confirms the desired inactive conformations. Selectivity profiling across a diverse kinome panel yields insight into the divergent selectivity of conformation-selective inhibitors. Notably, these inhibitors represent the first 'matched set' of conformation-selective kinase inhibitors that share the same hinge-binding scaffold and have been verified crystallographically. Using this unique

inhibitor set, our results provide a deeper understanding of kinase inhibitor structure–function and selectivity.

RESULTS AND DISCUSSION

Design and structural characterization of DFG-out dasatinib analogs.

To design an analog of dasatinib that invokes the DFG-out conformation, we applied a hybridization strategy developed by Gray and colleagues for converting Type I kinase inhibitors into Type II inhibitors.^{32,35} Specifically, we created an overlay of dasatinib bound to c-Src (PDB: 3G5D) with imatinib bound to c-Src (PDB: 2OIQ) (Figure 1A). The overlaid structures suggested that addition of a benzamide group to the meta-position of dasatinib's distal phenyl could result in a potent Type II analog of dasatinib. Following this design strategy, we synthesized two putative Type II inhibitors: **DAS-DFGO-I** and **DAS-DFGO-II**. These compounds vary only in the inclusion of a so-called 'flag methyl', a methyl group found both in dasatinib and in many Type II kinase inhibitors,⁷ including imatinib.

Dasatinib is a highly potent inhibitor of both c-Src and c-Abl ($K_i < 1$ nM). In activity assays, both **DAS-DFGO-I** and **DAS-DFGO-II** titrated the lowest enzyme concentration that can be used in this assay ($K_i < 1$ nM for both Type II analogs; Supplemental Figure S1). To confirm that these Type II analogs invoke the DFG-out conformation, we obtained a crystal structure of **DAS-DFGO-I** bound to c-Src kinase domain (PDB: 4YBJ, Figure 1B). Several dasatinib:c-Src interactions were conserved in this crystal structure, including hydrogen bond contacts to the hinge and gatekeeper residues of c-Src (Figure 1C). Gratifyingly, c-Src is in the DFG-out inactive conformation when bound to **DAS-DFGO-I**, highlighted by the outward flip of Phe-405. The benzamide group we appended to dasatinib occupies the DFG-pocket, with the amide hydrogen bonding to Glu-310 and Asp-404, all of which are prototypical features of Type II inhibitors. Notably, a previously obtained crystal structure of imatinib bound to c-Src illustrates an identical, DFG-out binding mode (Supplemental Figure S2).

Design and structural characterization of α C-helix-out dasatinib analogs.

While design strategies for the conversion of Type I kinase inhibitors into Type II inhibitors are known,^{32,35} no such strategy has been proposed for the conversion of Type I inhibitors into α C-helix out (CHO) binding analogs. Thus, we first analyzed the binding space occupied by existing CHO inhibitors that have co-crystal structures available (for a list of CHO inhibitors used, see Supplemental Table S1).³⁶ In contrast to Type II inhibitors, CHO inhibitors typically contain a bulky substituent that extends directly toward the α C-helix and lack hydrogen bond donors typically found in Type II inhibitors. Indeed, phenoxy or benzyloxy groups are commonly employed in the design of CHO inhibitors.^{12,37,38} An overlay of dasatinib:c-Src (PDB: 3G5D) with a panel of known and verified CHO inhibitors (Figure 2A), suggested that addition of a para-phenoxy group to dasatinib could lead to an optimal CHO analog of dasatinib. Thus, we synthesized two putative CHO inhibitors: **DAS-CHO-I** and **DAS-CHO-II**, where **DAS-CHO-II** incorporates the 'flag methyl' found in dasatinib and **DAS-DFGO-II**.

Consistent with dasatinib and the DFG-out analogs of dasatinib, both **DAS-CHO-I** and **DAS-CHO-II** are exceptionally potent in biochemical activity assays against both c-Src and c-Abl (Supplemental Figure S3). To confirm the proposed CHO conformation, we obtained a crystal structure of **DAS-CHO-I** bound to the c-Src kinase domain (Figure 2B; PDB: 4YBK). c-Src bound to **DAS-CHO-I** adopts the classic CHO (also termed Src/Cdk-like) inactive conformation^{8–12}, as evidenced by the outward rotation of the α C-helix and outward projection of Glu-310 side chain from the ATP pocket. The phenoxy group cannot be accommodated within the DFG pocket and thus occupies the empty space that results from the outward movement of the α C-helix. As with the DFG-out analog, the hinge contacts are conserved and the Type I portion of the molecule bears striking similarity to dasatinib's binding mode. In the c-Src:**DAS-CHO-I** structure the α C-helix is rotated nearly 5 Å from the active conformation of c-Src (Figure 2C), a change consistent with the binding of other CHO inhibitors to c-Src.^{11,12}

Recent evidence has suggested that many kinases can adopt the DFG-out inactive conformation, however, relatively little is known about which kinases can adopt the CHO inactive conformation. Because our results showed that c-Abl was potently inhibited by **DAS-CHO-I**, we obtained a crystal structure of this CHO inhibitor bound to c-Abl (Figure 2D). In this structure, there is less movement of the α C-helix (Figure 2E), however, the key lysine-glutamate salt bridge is disrupted, which is a unique feature of CHO inhibitors. A recently reported structure of a CHO inhibitor bound to Erk2 possesses the same features (minimal movement of α C-helix and disruption of Lys-Glu salt bridge).³⁸ Notably, our structure is the first of c-Abl bound to a small molecule inhibitor that binds the CHO inactive conformation.³⁹

Characterization of conformation-selective dasatinib analogs binding to target kinases.

Due to the constraints of our activity-based assays for c-Src and c-Abl (the main targets of dasatinib), we used the KINOMEScan service⁴⁰ to obtain quantitative K_d values for both c-Src and c-Abl (Table 1). Each compound is a very potent inhibitor of both c-Src and c-Abl without discrimination between targets.

We also examined the ability of our probes to bind to c-Abl that has been phosphorylated on the activation loop. Largely on the basis of imatinib's strong preference for non-phosphorylated c-Abl,^{21,41,42} an often reported "feature" of Type II inhibitors is that they bind c-Abl tighter than c-Abl-pY393.^{26,35,43} Given this frequently claimed feature, we were surprised to see that both **DAS-DFGO-I** and **DAS-DFGO-II** have no preference between the activation state of the kinase (Table 2). To follow up on these results, we screened a panel of bona fide Type II inhibitors of c-Abl (imatinib, nilotinib, rebastinib, and ponatinib) for which a crystal structure verifies the DFG-out inactive conformation,^{41,44–46} and found that only imatinib has a preference for c-Abl over c-Abl-pY393. Imatinib's hydrophobic hinge binding functionality has been hypothesized to be responsible for its preference for non-phosphorylated c-Abl.^{24,26} From these studies, we can state that Type II kinase inhibitors do not have an inherent activation-state preference in biochemical assays and this type of measurement cannot be relied upon to determine inhibitor binding mode, despite claims to the contrary.^{35,43}

In general, Type II inhibitors have been reported to have longer residence times, compared to Type I kinase inhibitors.^{21,24,47–49} However, these studies have not been performed using a controlled set of inhibitors, such as our dasatinib analogs. Thus, we synthesized BODIPY-labeled analogs (Figure 3A) for four of our dasatinib analogs (**DAS-**, **DAS-DFGO-I-**, **DAS-DFGO-II**, and **DAS-CHO-II-BODIPY**; for all probe data with wt c-Src and c-Abl, see Supplemental Figure S4). We first obtained K_d values for each probe and verified that binding was not impacted by addition of BODIPY-FL on the solvent-exposed piperazine ring. We then determined off-rates for each probe with both c-Src and c-Abl kinase domains. Consistent with previous reports, our Type II probes have significantly longer half-lives compared to the active-binding analog with both c-Src and c-Abl (Figure 3B). Relatively little is known about the binding kinetics of CHO kinase inhibitors, however, lapatinib (an FDA-approved CHO kinase inhibitor), has an exceptionally long half-life with its primary target EGFR ($t_{1/2} = 300$ min).³⁷ We found that **DAS-BODIPY** and **DAS-CHO-II-BODIPY** have nearly identical off-rates (Supplemental Figure S4) for both c-Src and c-Abl, suggesting that a slow off-rate is not an inherent feature of CHO kinase inhibitors.

A significant amount of effort has been spent on understanding the binding preference of imatinib for c-Abl over c-Src.^{21,50,51} Initial reports suggested that c-Src was unable to adopt the DFG-out conformation (a hypothesis that has since been clearly refuted).²² Despite evidence to the contrary,^{23–26} the belief that there is an energetic penalty for c-Src to invoke the DFG-out conformation persists.^{52–54} Our probes represent a unique opportunity to examine whether there is any penalty (kinetic or thermodynamic) for the DFG-out conformation in c-Src. Using **DAS-BODIPY** and **DAS-DFGO-II-BODIPY**, we compared the binding of each probes to both c-Src and c-Abl kinase domains. With **DAS-BODIPY**, we observed no significant change in kinetics (k_{on} and k_{off}) or thermodynamics (K_d) of binding (Figure 3C). Likewise, we observed no significant change in the kinetics and thermodynamics for binding of **DAS-DFGO-II-BODIPY**. We also observed no significant differences between c-Src kinase domain and full-length constructs. From these data, we conclude that imatinib's preference for c-Abl over c-Src is inherent to the ligand structure of imatinib and not conserved between Type II inhibitors.²⁴

To complement our thermodynamic data (K_d values, Table 2) that suggest Type II inhibitors do not have a preference for a specific kinase activation-state, we examined the kinetics of **DAS-BODIPY** and **DAS-DFGO-II-BODIPY** for binding to c-Src~pY416 (activation-loop phosphorylated c-Src kinase domain). We found no significant change in the binding of either inhibitor (Supplemental Figure S5). These data reinforce our findings that conformation-selective inhibitors do not have an inherent activation-state preference in biochemical assays.

Selectivity profiling of conformation-selective dasatinib analogs.

Type II inhibitors have been repeatedly stated to be more selective, in general, than Type I inhibitors.²⁹ These analyses hinge upon the profiling of a collection of unrelated kinase inhibitors. Recent analyses have pointed out that several previously profiled Type II inhibitors are highly promiscuous,⁷ however, kinome-wide profiling of a matched set of conformation-selective kinase inhibitors has not been reported. Thus, we wanted to

determine how the selectivity of dasatinib, a relatively promiscuous Type I inhibitor, compares to our panel of conformation-selective dasatinib analogs. We used a commercially available profiling service^{55,56} to profile each of the 5 analogs using a competition-binding assay for 124 non-mutant kinase domain constructs (Figure 4A; for individual kinase data, see Supplemental Data). We selected 500 nM as the screening concentration for each analog, a concentration that is significantly higher than each analog's binding affinity for c-Src and c-Abl, the primary targets of dasatinib. Kinome-wide selectivity differences between the 5 compounds can be compared using their S -score. S -score represents the fraction of kinases inhibited to a particular level (e.g., S_{35} is the number of kinases with <35% of control activity divided by the total number of nonmutant kinases in the panel, here 124). In this panel, dasatinib has $S_{35} = 0.21$ at 500 nM. The Type II analogs (**DAS-DFGO-I** and **DAS-DFGO-II**) have similar S_{35} scores (0.24 and 0.20, respectively). Meanwhile, the CHO analogs are significantly more selective (**DAS-CHO-I** $S_{35} = 0.05$, **DAS-CHO-II** $S_{35} = 0.16$). It is worth noting that these selectivity scores do not correlate to inhibitor potency for an individual target (e.g., **DAS-CHO-I**, ($S_{35} = 0.05$) binds LIMK1 more potently than dasatinib ($S_{35} = 0.21$), see Supplemental Data for details).

This is the first kinome-wide selectivity analysis for a matched set of conformation-selective kinase inhibitors. Our findings suggest that conversion of a Type I inhibitor into a Type II inhibitor leads to similar or even reduced selectivity. This is in stark contrast to the dogma that Type II inhibitors are inherently more selective than their Type I counterparts.²⁹ Of interest, is that while dasatinib is a poor inhibitor of non-Thr gatekeeper kinases, the Type II inhibitors (**DAS-DFGO-I** and **DAS-DFGO-II**) can potently inhibit many non-Thr gatekeeper kinases (Figure 4B). We confirmed this with by obtaining K_d values using an orthogonal assay (KINOMEscan K_d determinations) and found that several non-Thr gatekeeper kinases can be preferentially inhibited by the DFG-out analogs (Supplemental Table S2). Notably, there are no kinases (in this panel) that bind dasatinib and do not also bind to the Type II analogs. Thus, the ability of the DFG-out analogs to bind kinases with non-Thr gatekeeper residues is responsible for the observed slight loss of selectivity compared to dasatinib. On the basis of the similar selectivity obtained for all Type I and Type II analogs, the hinge binder is primary determinant for selectivity with these analogs. This has two important implications: 1) These data are in agreement with recent analyses that proposed that the DFG-out conformation is conserved across the kinome,⁷ and 2) Kinome-wide selectivity analyses using inhibitor sets are likely driven by the selectivity of the hinge binder.

Interestingly, there have been recent publications claiming that the gatekeeper residue is a primary determinant for a kinase's ability to bind a Type II inhibitor.⁵⁷ Specifically, it has been proposed that non-Thr gatekeeper residues can prevent a kinase's ability to bind a Type II inhibitor. Our data clearly demonstrates that kinases with non-Thr gatekeeper residues can be inhibited by Type II inhibitors. In fact, in our panels many such kinases (e.g., FLT1, RET, TIE2) are only inhibited by the Type II analogs (Figure 4).

We found that both of the dasatinib CHO analogs (**DAS-CHO-I** and **DAS-CHO-II**) are more selective than dasatinib. Several kinases are bound potently by both dasatinib and the CHO analogs (e.g., c-Src, c-Abl, LIMK1), however, there are many kinases that dasatinib

potently binds to which the CHO analogs cannot bind (e.g., EPHB2, PDGFRA, and TXK) (Figure 4B; Supplemental Table S3). The inability of certain kinases, including the entire Ephrin family, to bind the CHO inhibitors is responsible for the increase in selectivity for the CHO analogs. We did not identify any kinases that can accommodate the CHO analog that do not also potently bind the Type I analog. These results are intriguing and suggest that invoking the CHO inactive conformation could be a useful method to improve inhibitor selectivity, provided that the target kinase can adopt this inactive conformation. Furthermore, it suggests that the CHO inactive conformation is less conserved across protein kinases than the DFG-out inactive conformation (in contrast to the DFG-out conformation which is conserved kinome-wide).

Since the FDA-approval of imatinib in 2001, extensive effort has focused on the development of kinase inhibitors that target the inactive conformation(s) of kinases. On the basis of imatinib's binding properties, many (incorrect) theories about kinase inhibitor were established, many of which are still widely believed.²⁹ In an effort to evaluate how kinase inhibitor binding mode impacts selectivity, and target binding, we designed and characterized the first set of kinase inhibitor analogs that bind the active and inactive conformations (DFG-out and α C-helix out).

We have demonstrated that conversion of dasatinib to a Type II analog can decrease kinome-wide selectivity. The diminished selectivity observed with the Type II binding mode is a result of these analogs potently inhibiting non-Thr gatekeeper kinases (in contrast to the Type I analog). We also demonstrate that Type II inhibitors do not inherently prefer to bind the un-activated kinase state in biochemical assays. Our series of inhibitors represent the first set of kinase inhibitors that bind the active, DFG-out inactive, and α C-helix out conformations and represent a useful compound series to interrogate how kinase conformation impacts inhibitor binding and selectivity.

MATERIALS AND METHODS

Inhibitors

Dasatinib was purchased from LC Labs. **DAS-DFGO-I**, **DAS-DFGO-II**, **DAS-CHO-I**, **DAS-CHO-II**, **DAS-BODIPY**,⁵⁸ **DAS-DFGO-I-BODIPY**, **DAS-DFGO-II-BODIPY** and **DAS-CHO-II-BODIPY** were synthesized as described in the Supplemental Data and prepared as 10 mM DMSO stock solutions

Protein Purification and Crystallization Conditions

Wild type chicken c-Src kinase domain (residues 251–533) and human c-Abl kinase domain (residues 229–512) in pET28a, modified with a TEV protease cleavable N-terminal 6x-His tag were provided by M. Seeliger (SUNY, Stony Brook) and J. Kuriyan (UC Berkeley). Cell growth and expression and protein purification were performed using modified literature protocols for expression of wild-type c-Src and c-Abl kinase domain.⁵⁹ Briefly, the plasmid containing the kinase (pET28a) was transformed into *E. Coli* BL21DE3 cells already containing YopH phosphatase (pCDFDuet-1 plasmid) and plated on LB agar with kanamycin (50 μ g/mL, kan) and streptomycin (50 μ g/mL, SM) and grown overnight at 37

°C. A single colony was picked for a starter culture (LB containing kan/SM) and subsequently grown overnight at 37 °C. The next day, scaled up cultures (TB containing kan/SM) were grown to an OD_{600nm} of 1.2 at 37 °C and cooled to 18 °C with shaking prior to induction for 16 h at 18 °C with 0.2 mM IPTG. Cells were harvested by 20 min centrifugation at 4,000 rpm at 4 °C and resuspended in 50 mM Tris (pH 8.0), 500 mM NaCl, 5% glycerol, 25 mM imidazole (buffer A) supplemented with 0.1 mM PMSF for immobilized Ni metal affinity chromatography. Cells were lysed via sonication and insoluble protein and cell debris was sedimented through a 50-min centrifugation at 14,000 rpm at 4 °C. The clear supernatant was loaded onto the Ni-NTA agarose affinity column (batch, QIAGEN) column. The resin was washed with 50 column volumes of buffer A and five column volumes (3X) of buffer QB (20 mM Tris, pH 8.0, 5% glycerol, 1 M NaCl). Protein was eluted with five column volumes (4X) of buffer B (buffer A plus 0.5 M imidazole). The buffer B fractions were pooled together and cleaved with 0.1 mg of TEV per 2.5 mg of crude kinase. Cleaved protein was loaded onto an anion exchange column (Q Sepharose, Fast Flow, GE Healthcare Life sciences) equilibrated with buffer QA (buffer QB without 1 M NaCl). Proteins were eluted with a linear gradient of 0–35% buffer QB and peak fractions were combined (based on kinase activity assay) and concentrated down in order to be loaded onto a size-exclusion column (HiLoad Superdex S75, GE Healthcare Life sciences) equilibrated with buffer D (50 mM Tris, pH 8.0, 5% glycerol, 200 mM NaCl, 1 mM DTT). Pure fractions via SDS-PAGE were combined and concentrated down to a concentration of ~8 mg/mL (~260 μM).

Kinase-inhibitor complexes were formed in solutions of 163 μM kinase domain, 250 μM inhibitor, 50 mM Tris (pH 8.0), 200 mM NaCl, 5% (v/v) DMSO and 5–6% (v/v) glycerol. Crystals grew overnight at 20 °C using hanging-drop vapor diffusion. Drops contained 1 μL of complex + 1 μL well solution (0.1 M MES (pH 6.5), 16–22% (w/v) PEG 3350 and 100–420 mM sodium acetate). Prior to data collection, crystals were cryoprotected in well solution plus 20% (v/v) glycerol. X-ray diffraction data for both c-Src complexes were collected at 100 K and at a wavelength of 0.9787 Å at the Advanced Photon Source at Argonne National Laboratories on the 21-ID-F beamline. Data for the c-Abl complex was collected at 100 K and at a wavelength of 0.9793 Å on the 21-ID-D beamline.

Structure Determination of Kinase-Inhibitor Complexes

For the three protein-inhibitor complexes, diffraction data were processed and scaled with HKL2000.⁶⁰ The structures were solved by molecular replacement using PHASER.⁶¹ The inactive conformation of the kinase domain of chicken c-Src (PDB code 2OIQ²¹; residues 260–520 and PDB code 4DGG¹²; residues 266–519) without the αC-helix (residues 298–310) and the activation loop (residues 400–425) were the search models for the Src–**DAS-DFGO-I** and Src–**DAS-CHO-I** structures, respectively. The inactive conformation of the kinase domain of human c-Abl (PDB code 2G1T³⁹; residues 232–502) without the αC-helix (residues 275–287) and the activation loop (residues 377–402) was the search model for the Abl–**DAS-CHO-I** complex. The structures were built with iterative rounds of electron density fitting in Coot⁶² and refinement in autoBUSTER.⁶³ After the initial refinement, clear Fo-Fc density was visible for each compound in the ATP pocket of the protein. Three-dimensional coordinates and restraint files for each ligand were generated using smiles

string in GRADE⁶⁴ employing the mogul+qm option. The structures were analyzed with Molprobit⁶⁵ and EDS server.⁶⁶ Results are listed in Supplemental Data.

BODIPY Probe K_d Determination

An endpoint fluorescence assay⁴⁹ was used to determine K_d values. Reaction volumes of 50 μ L were used in 96-well plates. 34 μ L of buffer A 1X (100 mM Tris pH 8, 10 mM $MgCl_2$) was added to a single row, followed by 15 μ L of enzyme (3.3X concentration) in buffer A with 3-fold dilutions (typically 125 nM, 42, 14, 4.6, 1.5, 0.5, 0.17, 0.06, 0.02, 0.01 and 0 μ M final well concentration in buffer A). Then 1 μ L of a 500 nM stock of the appropriate dasatinib analog BODIPY probe in DMSO was added (2% DMSO final). Wells were incubated at rt for 30 minutes prior to end point read (ex/em 485/535 nm). Reactions had final concentrations of 10 nM BODIPY-probe, 100 mM Tris buffer (pH 8) and 10 mM $MgCl_2$. For K_d determination, the values were obtained directly from the nonlinear regression one-site binding curves (using normalized data) in the presence of various concentrations of the enzyme. The equation $Y = (B_{max} * X)/(K_d + X)$; was used in the nonlinear regression. All 4 runs were averaged together for each reported value.

Inhibitor Off-rate Determination

A multiple time point read fluorescence assay⁴⁹ was used to determine dasatinib analog BODIPY off-rates. Briefly, 60 μ L of total volume with 700 nM enzyme and 500 nM probe in buffer A 1X (Master Mix) was incubated at rt for 4 h along with 60 μ L of 500 nM probe alone in buffer A 1X (Blank Mix). Following this incubation period, 4 μ L of the master mix was added into 5 wells and 4 μ L of the blank mix was added into 2 wells via multichannel pipette into 116 μ L of buffer A 1X containing 5 μ M (final concentration) unlabeled dasatinib (120 μ L total, 30-fold dilution). Additionally, 4 μ L of master mix was added into a single well of 116 μ L of buffer A 1X containing 100 nM (final concentration) probe to maintain consistent plate reader gain values over the course of the fluorescent reads. Master mix dilutions with competitor had final concentrations of 23 nM enzyme, 17 nM BODIPY-probe, 5 μ M unlabeled dasatinib, 100 mM Tris buffer pH 8 and 10 mM $MgCl_2$. Reads (ex/em 485/535 nm) were taken every 10 minutes for the first 2 h, then every 20 min for next two hours and finally every 30 min for the remainder of the assay (12 h total). The values for k_{off} determination were obtained directly from the nonlinear regression fits for one-phase decay curves (using blanked data). The equation $Y = (Y_0 - Plateau) * \exp(-K * X) + Plateau$; was used in the nonlinear regression. An average of 5 wells at each time point was utilized for the final fit values produced.

Kinome-wide Inhibitor Profiling

Inhibitor selectivity profiles were obtained through Luceome Biotechnologies (Tuscon, AZ). Each inhibitor was screened at 0.5 μ M against a panel of 124 wt kinases.^{55,56}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Overlay of dasatinib (blue) and imatinib (red) kinase inhibitors used in the design of dasatinib DFG-out analogs, **DAS-DFGO-I** and **DAS-DFGO-II**. (B) **DAS-DFGO-I** (white) bound to c-Src (PDB: 4YBJ). P-loop (blue), α C-helix (yellow) and DFG motif (red) are shown. (C) The trifluoromethyl benzamide group is positioned within the DFG pocket, resulting in the DFG-out, inactive kinase conformation.

Figure 2.

(A) Overlay of dasatinib (blue) with the binding space (yellow) of structurally confirmed CHO inhibitors. A para-phenoxy group addition to dasatinib led to the generation of **DAS-CHO-I** and **DAS-CHO-II**. (B) **DAS-CHO-I** bound to c-Src (PDB: 4YBK). (C) An overlay with dasatinib bound to c-Src (PDB: 3G5D) shows the magnitude of the α C-helix shift (~5 Å) and incompatible binding between the CHO inhibitor and the Glu310 side chain of the kinase. (D) **DAS-CHO-I** bound to c-Abl (PDB: 4YC8). (E) The possible range of α C-helix positioning, from α C-helix in (blue) to c-Abl α C-helix out (green) to c-Src α C-helix out (yellow).

Figure 3.

(A) **DAS-** and **DAS-DFGO-II-BODIPY** structures. (B) Determination of BODIPY probes half-life values. A significantly longer half-life measurement was observed for **DAS-DFGO-II-BODIPY** (red) in comparison to **DAS-BODIPY** (blue) (C) Kinetic and thermodynamic data comparison for type I and type II BODIPY probes.

Figure 4.

(A) Kinase target comparison for conformation-selective dasatinib analogs utilizing a 50% cutoff value. (B) Analysis of kinase targets amongst the three conformation-selective modes of inhibition. Twenty kinases are targeted equally by the inhibitor set, while six kinases demonstrate a preference for DFG-out binding. Seventeen kinases show shared inhibition between dasatinib and the two DFG-out inhibitors. (C) Kinome tree representation of kinase targets. Colors correspond to the Venn diagram.

Table 1.KINOMEScan K_d profiling of conformation-selective dasatinib analogs.

Inhibitor	binding mode	R_2	Binding Mode:	
			active	DFG-out
Dasatinib	active	Me	0.07 ± 0.01	0.03 ± 0.01
DAS-DFGO-I	DFG-out	H	0.53 ± 0.01	2.2 ± 0.3
DAS-DFGO-II	DFG-out	Me	2.7 ± 0.01	2.9 ± 0.05
DAS-CHO-I	CHO	H	15 ± 3	14 ± 1
DAS-CHO-II	CHO	Me	1.7 ± 0.5	0.32 ± 0.02

Table 2.

Type II inhibitor phosphorylation state dependence comparison

Inhibitor	c-Abl K _d , nM	c-Abl-pY393 K _d , nM
DAS-DFGO-I	2.2 ± 0.3	1.8 ± 0.2
DAS-DFGO-II	2.9 ± 0.05	2.0 ± 0.01
imatinib	1.5 ± 0.2	24 ± 1
nilotinib	11 ± 2	15 ± 4
rebastinib	7.2 ± 0.1	11 ± 5
Ponatinib	0.7 ± 0.1	0.7 ± 0.1