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Bivalent inhibitors of c-Src tyrosine kinase that bind a regulatory domain

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Abstract

We have developed a general methodology to produce bivalent kinase inhibitors for c-Src that interact with the SH2 and ATP binding pockets. Our approach led to a highly selective bivalent inhibitor of c-Src. We demonstrate impressive selectivity for c-Src over homologous kinases. Exploration of the unexpected high level of selectivity yielded insight into the inherent flexibility of homologous kinases. Finally, we demonstrate that our methodology is modular and both the ATP-competitive fragment and conjugation chemistry can be swapped.

INTRODUCTION

Protein kinases are key regulatory enzymes in human cell signaling, both in healthy and diseased tissue. Efforts to pharmacologically elucidate kinase signaling have been hampered by a lack of selective kinase inhibitors.^{1–3} The vast majority of reported kinase inhibitors bind to the conserved ATP-site.⁴ Owing to the conserved nature of the ATP pocket, nearly all ATP-competitive kinase inhibitors are promiscuous and bind to undesired off-targets.^{4–6} One strategy to improve the selectivity of kinase inhibitors is to interact with elements outside the conserved ATP pocket.^{7–9}

We previously reported bisubstrate inhibitors of c-Src in which an ATP-competitive inhibitor was tethered to a substrate phosphorylation site peptide.¹⁰ Here, we explore the conversion of a promiscuous ATP-competitive kinase inhibitor into a bivalent inhibitor of c-Src that interacts with the SH2 domain of c-Src (in addition to the ATP-binding pocket). Our strategy involves conjugation of the two binding elements with “click chemistry” to enable a modular design.¹¹ We demonstrate that both copper-catalyzed and catalyst-free strain-release click reactions¹² can readily be employed to construct bivalent inhibitors. The modularity of our design is showcased by the synthesis of two distinct bivalent inhibitors starting from two different ATP-competitive inhibitor fragments.

Bivalent kinase inhibitors that target two distinct domains have been reported,^{8,9} however, there are no reports of such bivalent inhibitors purely constructed of small molecule—peptide hybrids. In addition, little is known about the selectivity changes transitioning from a

promiscuous ATP-competitive inhibitor to a bivalent inhibitor also targeting a regulatory domain (here SH2 domain).¹³ Lawrence and co-workers reported fully peptidic bivalent inhibitors that link a substrate-phosphorylation site peptide to a SH2 domain interacting peptide.^{14,15} Maly and co-workers have reported linking ATP-competitive small molecules to SH2 domain binding peptides using a large, protein-based linker.¹⁶ In contrast, our design provides a significantly lower molecular weight bivalent inhibitor than either method previously described.

c-Src is known to exist in two distinct global conformations, open and closed (Figure 1).^{17,18} The open conformation is the conformation that exists associated with the plasma membrane and is known to correlate with disease progression.¹⁹ In contrast to the open conformation, the closed conformation is highly rigid.²⁰ Crystal structures of the closed conformation reveal that the distance between the ATP-pocket and the phospho-Tyr binding pocket of the SH2 domain is 37 Å (Figure 1 and Supplementary Figure S1). Due to the inherent rigidity of the inactive conformation, we hypothesized that we could selectively inhibit the open conformation using a bivalent inhibitor constructed with a linker shorter than 37 Å. We predicted that a bivalent inhibitor with a linker too short to interact with the closed, rigid conformation could still bind to the open, flexible conformation. To our knowledge, there has been no report of a c-Src inhibitor that can selectively inhibit the active conformation over the inactive conformation.

RESULTS AND DISCUSSION

Our design strategy involves ATP-competitive inhibitors with a pendant alkyne and a SH2 domain binding phospho-peptide containing a PEG linker and pendant azide (Scheme 1).^{21–23} We hypothesized that a linker shorter than 37 Å could provide an inhibitor that selectively inhibits the active conformation of c-Src. Thus, we elected to use a 33-atom PEG linker with a calculated maximal length of 25 Å (Supplementary Figure S2). For an ATP-competitive inhibitor, we selected an aminopyrazole fragment that is a promiscuous kinase inhibitor.²⁴ We previously profiled an aminopyrazole fragment and found that it potently binds 117 of 200 kinases in that panel.²⁵ To enable conjugation to a peptide, we synthesized **1**, an aminopyrazole fragment with a pendant alkyne. We found **1** to be a competent inhibitor of 3-domain c-Src ($IC_{50} = 2.9 \mu M$). For the SH2 domain interacting peptide, we selected a previously reported pentapeptide, H_2N -Q-pY-E-E-I-CONH₂.²⁶ Solid phase peptide synthesis was employed to synthesize **2**, the SH2 interacting peptide with an N-terminal azido linker.²⁷ Copper-mediated click chemistry was then used to construct bivalent inhibitor **3**.

We evaluated bivalent inhibitor **3** in a continuous activity assay²⁸ with 3-domain c-Src²⁹ and found **3** was a potent inhibitor (Table 1, $IC_{50} = 0.16 \mu M$). Relative to ATP-competitive fragment **1**, this represents a 18-fold improvement in binding affinity. We next tested **3** against a kinase domain only construct of c-Src and found that bivalent inhibitor **3** has similar affinity to the ATP-competitive inhibitor alone (**3**, $IC_{50} = 2.1 \mu M$; **1**, $IC_{50} = 1.3 \mu M$). These results are consistent with bivalent inhibitor **3** requiring both the catalytic and SH2 domains to achieve optimal binding. Comparing kinase domain to 3-domain c-Src constructs, bivalent inhibitor **3** binds 13-fold tighter to the c-Src construct with an SH2 domain (Table 1).

We hypothesized that using a linker shorter than the distance from the ATP-site to the SH2-domain found in the crystal structure for inactive/closed conformation of c-Src (PDB: 2SRC) we could selectively target the more flexible active conformation of c-Src. To test this hypothesis, we utilized constructs of 3-domain c-Src that are active (pY416)³⁰ and conformations known to be inactive and rigid (pY527 and SH2-engaged).^{17,31} Gratifyingly, we found that bivalent inhibitor **3** is a selective inhibitor of pY416 c-Src over the pY527 and SH2-engaged constructs (Table 1: pY416 IC₅₀ = 0.24 μM, pY527 IC₅₀ = 1.8 μM, SH2-engaged IC₅₀ = 2.3 μM). In contrast, ATP-competitive inhibitor **1** has no preference for open vs closed c-Src constructs (see Supporting Information for details). To our knowledge, there are no reports for any kinase inhibitor with this level of selectivity for the active kinase conformation.

Next, we wanted to determine whether bivalent inhibitor **3** is selective for c-Src over homologous kinases. Thus, we tested both ATP-competitive inhibitor **1** and bivalent inhibitor **3** for inhibition against a panel of 8 homologous kinases,³² including 7 members of the Src family (Table 2). Importantly, each of these kinases includes a SH2 domain.^{33,34} The average IC₅₀ for bivalent inhibitor **3** was 3.5 μM. From these data, we observe that **3** is highly selective (average of 22-fold selective) for c-Src across this panel of homologous kinases (Table 2). In addition, we observed only a modest increase in potency for bivalent inhibitor **3** compared to ATP-competitive inhibitor **1**.

The level of selectivity we observed across homologous kinases is very impressive, however, it was unexpected given that the ATP-competitive inhibitor inhibits each kinase similarly and the SH2 binding element is known to interact with many Src family kinases.^{26,35} We wanted to study the surprising selectivity using c-Src and Hck kinases. The ATP-competitive fragment binds with similar potency to both c-Src and Hck (c-Src IC₅₀ = 2.9 ± 0.6 μM; Hck IC₅₀ = 8.9 ± 0.5 μM) and cannot explain the selectivity observed for bivalent inhibitor **3**. Next, to probe the affinity of the SH2 interacting peptide with both c-Src and Hck, we measured the affinity of a related SH2 peptide containing a N-terminal fluorophore and the same core residues as our bivalent inhibitor (FITC-E-P-**Q-pY-E-E-I-P-I-Y-L-NH₂**, bold corresponds to the core SH2 binding residues found in bivalent inhibitor **3**). We found the SH2 peptide binds to both c-Src and Hck with equal affinity (c-Src EC₅₀ = 0.72 ± 0.1 μM, Hck EC₅₀ = 1.0 ± 0.3 μM). From these data, we conclude that the selectivity is not arising from either the ATP-competitive fragment or the SH2-binding element.

We hypothesized that the inter-domain flexibility for homologous kinases might be different – and thus responsible for the selectivity observed. To explore this hypothesis, we measured the binding of our bivalent inhibitor **3** to 3-domain Hck constructs with varied open/closed (flexible/rigid) conformations (in order of most open and flexible to most closed and rigid: wild-type, SH2-engaged, and SH3-engaged).^{36,37} We found that bivalent inhibitor **3** binds tighter to SH3-engaged compared to wild-type 3-domain Hck (IC₅₀ = 0.6 and 2.8 μM, respectively). These data suggest that activated Hck is less flexible than c-Src. Furthermore, engagement of the SH3 domain of Hck likely brings the SH2 and catalytic domains closer in proximity. Interestingly, Fyn1 is the only Src family kinase (SFK) other than c-Src for which **3** binds potently (Fyn1 IC₅₀ = 0.27 μM). A recent study of SFKs indicated that Fyn1 was significantly more flexible than other SFKs.³⁸ Together, our findings reveal that homologous

kinases have varied conformational flexibility, and that this flexibility can be selectively targeted using bivalent kinase inhibitors.

We have previously shown that bisubstrate kinase inhibitors are less susceptible to mutations within a single binding pocket.¹⁰ We wanted to determine whether bivalent inhibitor **3** could inhibit c-Src with a clinically relevant gatekeeper mutation (T338M).³⁹ The T338M mutation renders c-Src resistant to nearly all ATP-competitive inhibitors, including all FDA-approved inhibitors of c-Src.^{40,41} We found that bivalent inhibitor **3** is also potent inhibitor of 3-domain T338M c-Src ($IC_{50} = 0.19 \mu M$). These data suggest that bivalent inhibition of kinases is indeed an effective strategy to inhibit drug-resistant kinase mutants.

Our conjugation strategy, utilizing azide-alkyne click chemistry, was designed to be modular in nature. To showcase the modularity, we synthesized an analog of dasatinib (an FDA-approved dual c-Src/Abl inhibitor) with a pendant alkyne (see Supporting Information for details). Conjugation to the azido SH2 peptide **2** to dasatinib~alkyne (S11) yielded bivalent inhibitor **4** (Figure 2). This inhibitor was too potent to evaluate in our enzymatic activity assays (due to titration of the lowest enzyme concentrations accessible to our activity assay). Thus, we utilized a commercial binding assay that is performed in reticulocyte lysate containing ~5 mM ATP (Luceome Biotechnologies, Tucson, AZ)^{42,43} to measure the affinity of **4** with full-length c-Src. In this assay, compound **4** has an $IC_{50} = 1$ nM for full length Src, while the ATP-competitive fragment (dasatinib) has an IC_{50} of 18 nM. This represents an 18-fold improvement in binding affinity upon conversion of the ATP-competitive fragment to bivalent inhibitor **4**. Notably, an 18-fold improvement is identical to the binding affinity increase found with the aminopyrazole-based system. These data demonstrate that the ATP-competitive inhibitor can be swapped for a different ligand in a straightforward manner. Thus, one could modulate selectivity (or other properties) by selecting from a multitude of known ATP-competitive kinase inhibitors.

Finally, we wanted to explore whether we could replace the copper-catalyzed click conjugation chemistry with a strain-release click chemistry. To enable catalyst-free conjugations, we synthesized dasatinib with a pendant cyclooctyne (Scheme S4). Incubation of azido peptide **2** with dasatinib~cyclooctyne provided bivalent inhibitor **5** (Figure 2). In our enzymatic activity assay, this bivalent inhibitor titrates enzyme ($K_i < 10$ nM), having similar activity to the copper catalyzed version (**4**). One advantage of catalyst-free cycloadditions is the ability to perform on-enzyme conjugation in situations where copper might be toxic.^{12,44} This could enable rapid *in situ* generation of bivalent kinase inhibitors from starting fragments.

CONCLUSION

We have developed a platform to generate bivalent inhibitors that interact both with the ATP-binding site and a regulatory domain, here a SH2 domain. While the ATP pocket is conserved across the kinome,⁴ interaction with a SH2 domain can provide instant selectivity because the vast majority of kinases do not possess a SH2 domain.⁴⁵ Furthermore, we found that our ATP-to-SH2 bivalent kinase inhibitors possess high selectivity across homologous kinase. We found that selectivity can be obtained even for kinases which share optimal SH2-

peptide binding sequences. Using model kinases with varied flexibility, we demonstrated that flexibility of the kinase (in particular inter-domain flexibility) is responsible for the high level of selectivity obtained.

To demonstrate the modularity of our bivalent inhibitor design, we swapped the ATP-competitive fragment to a dasatinib analog and obtained a bivalent inhibitor with single digit nanomolar potency. The conjugation chemistry can also be changed, here we used both copper-catalyzed and strain-promoted click reactions to synthesize bivalent inhibitors.

While outside the scope of this manuscript, we have previously demonstrated that peptide-based kinase inhibitors can be used in cellular assays after appending a cell-penetrating peptide.¹⁰ In addition, one could envision using small molecule SH2 ligands to construct fully non-peptide bivalent inhibitors that might have inherent cell permeability. These studies are ongoing in our laboratory.

METHODS

Materials and experimental methods are described in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Conformational equilibrium of c-Src.

c-Src exists in equilibrium between 'open' and 'closed' conformations. The closed, autoinhibited conformation (left, PDB: 1Y57), and open, active conformations (middle, PDB: 2SRC, and right, PDB: 1OPL). The kinase catalytic domains are colored green, SH2 domains colored blue, and SH3 domains colored red. For each conformation, distances between the ATP-binding pocket and the SH2 phosphotyrosine binding pockets are displayed and colored gold.

Figure 2. Dasatinib-based bivalent inhibitors.

Copper catalyzed dasatinib bivalent **4** and catalyst-free bivalent **5**.

Scheme 1. Synthesis of bivalent inhibitor 3.

Copper catalyzed click cycloaddition of ATP-competitive alkyne **1** and azido-SH2-peptide **2** afford triazole-linked bivalent inhibitor **3**.

Table 1.IC₅₀ values for 1 and 3 with c-Src varied kinase constructs.

c-Src construct	1 IC ₅₀ (μM)	3 IC ₅₀ (μM)
kinase domain	1.3 ± 0.5	2.1 ± 0.3
3-domain (3D)	2.9 ± 0.6	0.16 ± 0.01
pY416 3D	2.4 ± 0.6	0.24 ± 0.1
pY527 3D	1.4 ± 0.1	1.8 ± 0.1
SH2-eng 3D	2.7 ± 0.6	2.2 ± 0.8

Table 2.

IC₅₀ values for 1 and 3 with c-Src varied homologous kinases.

kinase	1 IC ₅₀ (μM)	3 IC ₅₀ (μM)
Hck	8.9	2.8 ± 0.7
Lck	9.3	5.3 ± 0.6
Blk	21.8	5.3 ± 0.7
Frk	9.6	5.6 ± 0.4
Fyn1	2.8	0.27 ± 0.02
Fgr	4.6	3.1 ± 0.9
Lyn	10.8	2.5 ± 0.2
Abl	15.0	4.0 ± 0.4