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Exquisitely Specific Bisubstrate Inhibitors of c-Src Kinase

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Abstract

We have developed a modular approach to bisubstrate inhibition of protein kinases. We apply our methodology to c-Src and identify a highly selective bisubstrate inhibitor for this target. Our approach has yielded the most selective c-Src inhibitor to date, and methodology to render the bisubstrate inhibitor cell-permeable provides a highly valuable tool for the study of c-Src signaling. In addition, we have applied our bisubstrate inhibitor to develop a novel screening methodology to identify non-ATP-competitive inhibitors of c-Src. Using this methodology, we have discovered the most potent non-ATP-competitive inhibitor reported to date. Our methodology is designed to be general and could be applicable to additional kinases inhibited by the promiscuous ATP-competitive fragment used in our studies.

Reversible protein phosphorylation, mediated by protein kinases, is a vital posttranslational modification in eukaryotic cell signaling.^{1–3} These signaling networks are complex and efforts to understand these pathways has been hampered by a lack of selective kinase inhibitors.^{4–5} Nearly all kinase inhibitors bind within a highly conserved area of the kinase catalytic domain, the ATP-binding pocket.⁴ Thus, development of selective ATP-competitive kinase inhibitors is exceedingly challenging, and is often the result of serendipitous discovery.⁴ Non-ATP-competitive inhibitors possess higher degrees of selectivity, however, they generally suffer from a lack of potency.^{7–9}

Bisubstrate kinase inhibition, wherein the inhibitor interacts both with the ATP and protein substrate-binding sites, is an attractive strategy to gain selectivity while maintaining the high potency afforded via interactions within the ATP-binding pocket.^{9–12} Bisubstrate inhibitors of protein kinases have been of interest for some time, however, there are few examples where the potency and selectivity advantages are fully realized.^{9–12}

Herein, we report the development of modular protein kinase inhibitors that interact with both the ATP and protein substrate binding sites. In addition to the ability to tune the inhibitor to

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Supporting Information. Supplementary figures, experimental methods, and characterization of compounds 1–8. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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varied targets, we demonstrate remarkable potency and selectivity for the desired target. As proof of principle for our strategy we have developed bisubstrate inhibitors of the non-receptor tyrosine kinase c-Src, for which few selective probes are known.^{5,6}

Our modular strategy to bisubstrate kinase inhibitors utilizes a promiscuous ATP-competitive inhibitor that is then linked to a peptide derived from known substrates for the target kinase. We began by exploring the selectivity of an analog of PP2, a classic ATP-competitive inhibitor that is known to be highly promiscuous.⁵ In a panel of 200 diverse kinases, we found that compound **1** was able to tightly bind 26% (52) of the kinases, validating its use as a promiscuous ATP-competitive scaffold for our studies (Figure 1).

We began our studies by developing a bisubstrate inhibitor for the prototypical tyrosine kinase c-Src,^{13–14} which is strongly inhibited by promiscuous kinase inhibitor **1**. We envisioned the use of click chemistry to enable linkage of a c-Src peptide substrate to ATP-competitive inhibitor **1**. Thus, we synthesized compound **2**, a variant of inhibitor **1** where an alkyne is appended to the N1-phenyl (Figure 1). Next, we selected a consensus substrate sequence for c-Src (Ac-EEEIYGEFEA-NH₂) to serve as the substrate-competitive functionality of our bisubstrate c-Src inhibitor. To enable conjugation, the phosphorylatable tyrosine residue was replaced with 4-aminophenylalanine (4-NH₂-Phe). The peptide containing 4-NH₂-Phe was then acylated with an azide-containing linker.

The binding affinity of bivalent inhibitors that contain a linkage between two fragments capable of independent binding has previously been shown to be dependent upon the length of the linker.^{10,15–16} Thus, we explored several azido linkers with varied length and found an optimal length of 5 methylenes between the azide and carboxylic acid functionalities. This optimal linker length is in good agreement with molecular modeling that suggests a distance of ~11 Å between the attachment points of the two fragments (see Supplementary Figure S1). In biochemical assays, we found bisubstrate inhibitor **3** to be exceptionally potent (<30 nM IC₅₀ using 5 mM ATP and 45 μM peptide substrate). As expected, both shorter and longer linkers led to decreased binding affinity (Supplementary Table S1).

When performed correctly, bisubstrate inhibition should inherently lead to a synergistic increase in potency relative to both inhibitor fragments.¹⁰ However, this type of analysis is not always discussed in the literature and in many cases, the resulting bivalent inhibitor was shown to be a weaker binding than one of the initial fragments.¹⁰ To determine K_d values, we utilized a Cy5-conjugated analog of bisubstrate inhibitor **3**, the optimal bisubstrate inhibitor and utilized this in TR-FRET based assays.¹⁷ We obtained a K_d value of 0.28 nM for inhibitor **3**, while the ATP-competitive and substrate-competitive fragments have K_d values of 376 and 296 nM, respectively. Thus, our bisubstrate inhibitor **3** is 1,300-fold more potent than the ATP-competitive fragment **2** and 1,100-fold more potent than the substrate-competitive peptide fragment. These large fold increases represent some of the largest increases in binding affinity observed going from a monovalent fragment to a bisubstrate inhibitor,¹⁰ confirming that we identified an optimal linkage between the two fragments.¹⁶

Although not commonly validated in the literature, bivalent inhibitors of protein kinases should possess high degrees of selectivity.^{9–12} Upon identifying bisubstrate inhibitor **3** as one of the

most potent c-Src inhibitors known ($K_d = 0.28$ nM), we sought to characterize the selectivity of **3** in a large panel assay. We utilized a commercially available kinase panel of 213 kinases that measures competitive binding (KinaseSeekerTM, Luceome Biotechnologies).^{18,19} In this panel, we found only two kinases (c-Src and its close homolog c-Yes) were significantly bound by bisubstrate inhibitor **3** at 115 nM, a concentration >400-fold higher than compound **3**'s K_d for c-Src (Figure 2). Notably, no other kinase that was found to be bound by ATP-competitive fragment **2** was potently bound by **3**, including other Src family kinases. On the basis of these data, inhibitor **3** is the most selective inhibitor of c-Src reported to date ($S_{35}=0.01$).⁵⁻⁶

To interrogate the role of the substrate-competitive fragment of **3**, we synthesized bisubstrate inhibitor **4**, where the peptide fragment was replaced with a single 4-amino tyrosine residue to mimic the phosphorylatable tyrosine residue (Chart 1). Notably, inhibitor **4** is the lowest molecular weight bisubstrate inhibitor reported to date that merges ATP-competitive and substrate-competitive fragments.⁹⁻¹² Despite **4**'s lower molecular weight compared with traditional bisubstrate inhibitors, it is a potent inhibitor of c-Src ($K_d = 14$ nM). Unlike **3**, we found that **4** potently binds to kinases that are homologous to c-Src (see Supplementary Figure S3), confirming the important role of an extended peptide sequence to obtain selectivity.

In order for kinase inhibitors to serve as useful tools in biology, they must be cell-permeable.²⁰ Like all bisubstrate kinase inhibitors reported,⁹⁻¹² inhibitor **3** utilizes a peptidic substrate fragment, which limits cell-permeability (Figure 3). Notably, there are very few examples of bisubstrate kinase inhibitors used in cellular studies.⁹⁻¹² To render inhibitor **3** cell-permeable, we appended a commonly used poly-Arginine tag (Arg-9) to our bisubstrate inhibitor. Arg-9 tags have successfully been used to deliver many types of cargo to cells, including peptide-based inhibitors.²⁰⁻²⁴ We found that addition of an N-terminal Arg-9 tag had little impact on its biochemical affinity for c-Src (see Supporting Information), however, this construct (**7**) was cell permeable. Using an enzyme-linked immunosorbent assay (ELISA),⁵ we found that bisubstrate inhibitor **7** has an IC_{50} value of 0.9 μ M for cellular c-Src autophosphorylation. In addition, bisubstrate inhibitor **7** inhibits the growth of HT-29 and SKBR3 cell lines, which are both cancer cell lines that are growth dependent upon c-Src activity,²⁵ with comparable (or better) activity compared to PP2, the most commonly utilized c-Src inhibitor in cell biology (Figure 3).⁵ Unlike PP2, which is a promiscuous kinase inhibitor,⁵ **7** has no activity in c-Src growth independent cell lines MCF7 and T47D (see Supporting Information).²⁵ With cell-permeability and near perfect selectivity, bivalent inhibitor **7** is positioned to be a very useful chemical probe of c-Src activity.

We wondered if there might be any benefit to bisubstrate inhibition compared to standard ATP-competitive kinase inhibitors. In theory, bisubstrate kinase inhibitors should be well suited to bind resistance mutations due to the bisubstrate inhibitor's large interaction network with its target kinase. To test this hypothesis, we utilized c-Src with a mutation of the gatekeeper residue (T338I c-Src, see Supplementary Figure S4). We found that dasatinib, a clinically used dual c-Src/c-Abl kinase inhibitor, binds T338I c-Src >700,000-fold worse than WT c-Src (WT = 0.07 nM, T338I = 50 μ M). Meanwhile, bivalent inhibitor **3** binds T338I < 3,500-fold worse than WT c-Src (WT = 0.28 nM, T338I = 0.95 μ M). This type of analysis has not been previously

reported for a bisubstrate inhibitor and highlights that bisubstrate inhibitors are less sensitive to single-point mutations.^{9–12}

Because bisubstrate inhibitor **4** has significant interactions along the protein-binding surface of c-Src, we wondered if we could use our Cy5-labeled analog (**5**) to identify inhibitors of c-Src that are non ATP-competitive. Non-ATP-competitive kinase inhibitors have become of increasing interest, however, methods to identify non-ATP-competitive inhibitors are scarce.^{8,9} To validate that our bivalent probe can identify non-ATP-competitive inhibitors, we examined MEB-SCI, a substrate-competitive c-Src kinase inhibitor recently reported by our laboratory, and obtained a $K_d = 15.6 \mu\text{M}$, which is identical to the reported K_i value obtained using an activity-based assay.⁷ We envisioned that we could screen a library of putative non-ATP-competitive kinase inhibitors against our bisubstrate probe in a TR-FRET assay. A second screen against an ATP-competitive TR-FRET tracer removes any ATP-competitive compounds from the hits.¹⁷ We screened an in-house library of 300 small molecules that contain a privileged biphenyl scaffold and identified 14 compounds that displaced the bivalent tracer but not the ATP-competitive tracer. Activity assays were then used to characterize the modes of inhibition for each compound. From these screening data, we identified the most potent non-ATP-competitive inhibitor of c-Src reported to date (Figure 4, **6**, $K_d = 114 \text{ nM}$).⁸ Despite this screening methodology not having been reported previously, bisubstrate inhibitors are an ideal means to identify small molecule non-ATP-competitive inhibitors.⁸

Bisubstrate kinase inhibitors have been of great interest due to their promise for selectivity and potency.^{9–12} Herein, we described a modular approach to bivalent inhibitors and applied this methodology to c-Src. We demonstrated that our bisubstrate c-Src inhibitor is significantly more potent than either of the individual fragments. In addition, we demonstrated that the remarkable selectivity of our c-Src inhibitor comes from the extended peptide sequence and that we can modulate selectivity by altering the substrate-competitive peptide sequence. We demonstrate a route to cell-permeability enabling these highly selective and potent inhibitors to be used to study cell signaling processes. Finally using our bisubstrate inhibitor and novel screening methodology, we report the most potent non-ATP-competitive inhibitor of c-Src known to date. Our methodology is general and could potentially be applied to any of the 52 kinases targeted by ATP-competitive inhibitor **1**.

METHODS

Compound characterization, biochemical and cellular methods are found in Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of promiscuous ATP-competitive inhibitors **1** and **2**. Selectivity profile for compound **1** (10 μ M) against a panel of 200 kinases, determined using a binding assay (see supporting information for details). Red circles are indicative of inhibitor binding to a given kinase $> 35\%$ control. c-Src is highlighted in blue.

Figure 2. Synthesis of c-Src bisubstrate kinase inhibitor **3**. Selectivity profile for compound **3** (115 nM) against a panel of 213 kinases. determined using a binding assay (see supporting information for details). Red circles are indicative of inhibitor binding to a given kinase > 35% control. c-Src is highlighted in blue.

Figure 3. Structural overview of c-Src bisubstrate kinases inhibitor **3** and **7**. Structure of ATP-competitive kinase inhibitor **PP2**. Cell proliferation of **3**, **7**, and **PP2** with SKBR3 breast cancer cell line.

Figure 4. Structural overview of Cy5-tagged c-Src bisubstrate TR-FRET probe **5**. Structure of non-ATP-competitive c-Src kinase inhibitor **6**.

4**c-Src K_d = 14 nM**

Chart 1.
Structure of bisubstrate inhibitor **4**.