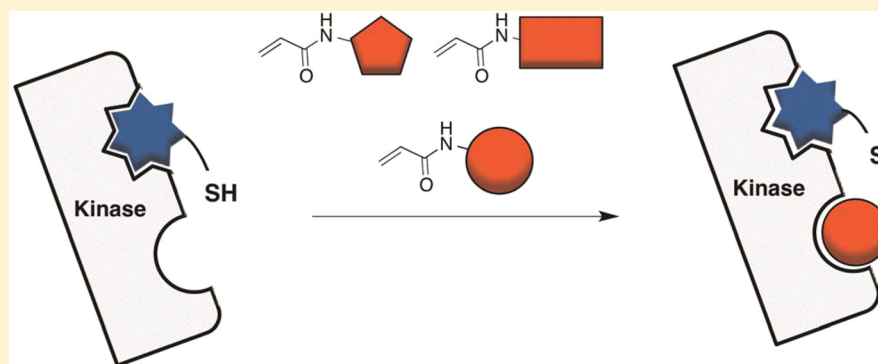


# Discovery of Bivalent Kinase Inhibitors via Enzyme-Templated Fragment Elaboration

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## Supporting Information



**ABSTRACT:** We have employed novel fragment-based screening methodology to discover bivalent kinase inhibitors with improved selectivity. Starting from a low molecular weight promiscuous kinase inhibitor, we appended a thiol for subsequent reaction with a library of acrylamide electrophiles. Enzyme-templated screening was performed to identify acrylamides that assemble into bivalent inhibitors of c-Src kinase. Upon identification of acrylamide fragments that improve the binding affinity of our lead thiol, we characterized the resulting bivalent inhibitors and identified a series of kinase inhibitors with improved potency and selectivity compared to the thiol-containing precursor. Provided that protein can be prepared free of endogenous reactive cysteines, our methodology is general and could be applied to nearly any enzyme of interest.

**KEYWORDS:** Kinase, selectivity, enzyme-templated

Protein kinases represent one of the largest protein families of the human genome and are responsible for regulating key signaling pathways in eukaryotes.<sup>1</sup> Aberrant protein phosphorylation within the cell has been implicated in numerous diseases and has subsequently made kinases attractive targets for medicinal chemistry.<sup>2–4</sup> All FDA-approved kinase inhibitors bind to the highly conserved ATP pocket due to the tractable targeting nature of this site.<sup>5</sup> Despite successful ATP-competitive inhibitors in the clinic, many inhibitors of this class suffer from poor selectivity profiles and subsequent off-target toxicity.<sup>6,7</sup> In an effort to improve inhibitor selectivity, features adjacent to or outside the ATP-binding site have been recently targeted.<sup>3,8</sup> The systematic discovery of inhibitors that bind outside the ATP site, however, is challenging and is often serendipitous.<sup>9,10</sup>

Fragment-based drug discovery (FBDD) has been successful for a number of targets, yet labor-intensive NMR or X-ray crystallography detection methods and ATP-pocket centric design strategies have limited its utility for kinases.<sup>11</sup> To apply the principles of FBDD to kinases in an unbiased manner without need for structural information, we developed a fragment elaboration screen where hits can be identified through an activity assay.

Traditional fragment tethering relies on mixed disulfide formation with the protein,<sup>12</sup> while our strategy involves a Michael reaction between an ATP-competitive hit compound with a pendent thiol and an acrylamide fragment. We benefit from using a proximity-based, enzyme-templated approach, where increases in local concentration of both reactive components leads to formation of a bivalent inhibitor in situ.<sup>13–15</sup> A major benefit of an enzyme-templated approach is the ability to use the same fragment library for diverse targets (as opposed to synthesizing and purifying bivalent inhibitors for each target). Bivalent inhibitors occupy two distinct binding sites connected by a linker. Importantly, bivalent inhibitors often have improved properties such as potency and/or selectivity.

We previously reported compound **1**, a highly promiscuous aminopyrazole kinase inhibitor.<sup>16</sup> We profiled **1** and found that **1** potently binds 117 of 200 kinases tested.<sup>15</sup> To enable enzyme-templated Michael reactions with a library of acrylamides, we synthesized an analogue of **1** that contains a

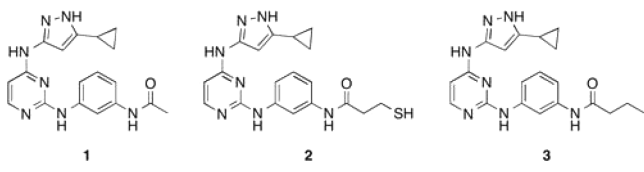
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thiol functionality (**2**) (Chart 1). Compound **3** was synthesized as a control absent a reactive thiol. As the electrophile

Chart 1. Structure of Promiscuous Kinase Inhibitors 1–3



component, acrylamides are attractive because they have low intrinsic reactivity.<sup>16,17</sup> Their relatively low reactivity will ensure that the screen will selectively identify enzyme-templated reactions. Conversely, electrophiles with high intrinsic reactivity (e.g., vinylsulfonamides) could produce conjugates in the absence of enzyme.<sup>17</sup>

We chose c-Src, a nonreceptor tyrosine kinase, as the initial target to develop our methodology. c-Src has been extensively studied over the years and implicated in a variety of diseases including the metastasis of several cancers,<sup>18,19</sup> and few selective inhibitors are available to study c-Src biology.<sup>5,8</sup> Importantly, compound **1** is a competent inhibitor of c-Src ( $K_i = 0.36 \pm 0.08 \mu\text{M}$ ). To ensure that our assay would identify only acrylamides that react with **2** and not c-Src itself, we made a mutant c-Src kinase with three nonessential, solvent-accessible cysteines mutated to serines on the surface of c-Src ("3M" c-Src: C277S, C483S, S496S). Of note, we found that compound **2** is a time-dependent, irreversible inhibitor of wild-type c-Src, but not of 3M c-Src.

A library of 110 acrylamide fragments (average molecular weight = 235 Da, see Supplemental Table S1 for structures) was incubated with 3M c-Src in the presence and absence of **2** (**2** was added at a concentration that approximates its  $\text{IC}_{20}$  value). After a 30 min incubation, we used a continuous activity assay to identify fragments that had significant inhibition difference (>2 standard deviations) between screens performed with and without thiol **2**. From the 110 acrylamide fragments, we identified four hits that met our criteria (Figure 1, hit rate of

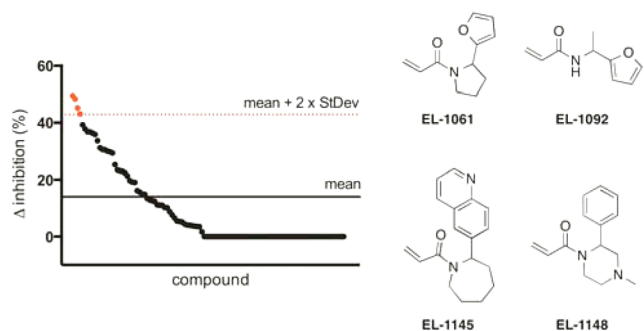


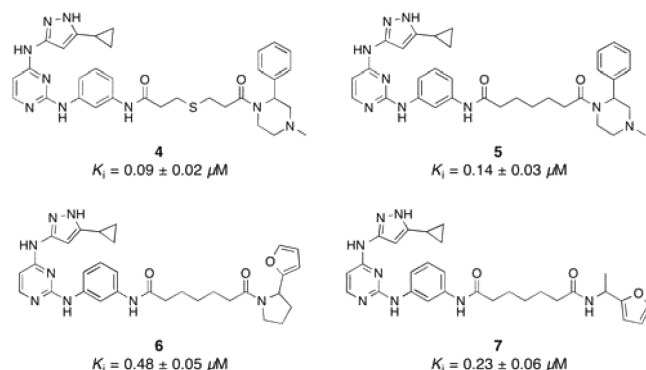
Figure 1. (left) Graph of enzyme-templated screen using c-Src and a library of 110 acrylamides. (right) Structures of acrylamide fragment hits.

3.6%). Each of the four hits was confirmed in three subsequent enzyme-templated validation screens (see Supporting Information for details and assay controls performed).

To demonstrate that the predicted enzyme-templated Michael additions were occurring, we performed a mass spectrometry analysis of thiol **2** + acrylamide **EL-1148** in the presence and absence of c-Src kinase. Only when c-Src was

present could we detect a mass for the Michael adduct (**4**). We next synthesized **4** and found it to have improved binding potency compared to **3** and **EL-1148**, the individual starting fragments (Chart 2). Because **2** is an irreversible inhibitor of

Chart 2. Structures of Bivalent Kinase Inhibitors



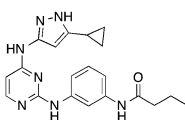
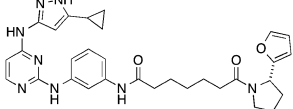
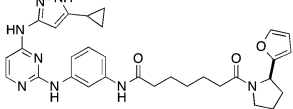
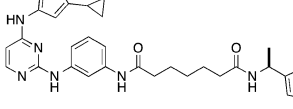
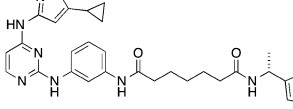
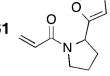
wild-type c-Src, we compared compound **3**, where the thiol of **2** is replaced with a carbon. Compound **3** ( $K_i = 0.40 \pm 0.02 \mu\text{M}$ ) was found to be less potent than bivalent inhibitor **4** ( $K_i = 0.09 \pm 0.02 \mu\text{M}$ ) for wild-type c-Src. To enable a comparison of thiol **2** with bivalent inhibitor **4**, we tested both for inhibition of 3M c-Src and found **4** to be a more potent inhibitor of this construct (**2**, 3M c-Src  $K_i = 0.20 \pm 0.06 \mu\text{M}$ ; **4**, 3M c-Src  $K_i = 0.12 \pm 0.02 \mu\text{M}$ ).

**EL-1148** is a very weak inhibitor of c-Src (3M c-Src,  $\text{IC}_{50} = 470 \pm 110 \mu\text{M}$ ) but was readily identified in our method. In traditional fragment-based screening, it could be difficult to identify weak binding fragments using activity-based assays and even more difficult to discriminate between ATP-competitive and non-ATP-competitive fragments. Using biochemical activity assays, we further demonstrated that **EL-1148** is noncompetitive with ATP, suggesting that it is binding in a pocket adjacent to the ATP-pocket (Supplemental Figure S1).

Because thioethers can undergo retro-Michael reactions in assay buffer conditions (and thus lose inhibitory potency), we synthesized **5**, which contains an all-carbon linker between the two fragments (Chart 2). Gratifyingly, **5** was able to inhibit c-Src with identical potency as **4**. We then synthesized bivalent inhibitors, again using an all-carbon linker, derived from the two furan-containing fragments (**EL-1061** and **EL-1092**) that were identified in our enzyme-templated screen (bivalent inhibitors **6** and **7**, respectively). In addition, because both **6** and **7** are a mixture of enantiomers, we synthesized both *R*- and *S*-enantiomers of the furan-containing amine fragments and subsequently produced enantiopure bivalent inhibitors. The amines were synthesized using Ellman sulfinylimine chemistry (see Supporting Information for synthetic details).<sup>20,21</sup> Upon characterization of the bivalent inhibitor series, we found that the bivalent inhibitors with *R*-stereochemistry had improved binding to c-Src (Table 1). A preference for a particular stereochemistry indicates specific binding to c-Src, despite the relatively weak binding observed with the acrylamide fragments.

Bivalent inhibitors **9** and **11** have 6- and 3-fold improvements in binding affinity for c-Src, respectively, compared to the control ATP-competitive fragment **3** (Table 1). We next sought to determine whether **9** and **11** have improved selectivity compared to fragment **3**. As a measure of selectivity, we assayed for inhibition against a small panel of homologous kinases (c-Yes, 95% similar to c-Src; Hck, 80% similar to c-Src;

**Table 1.**  $K_i$  Values for **1** and Enantiomerically Pure Bivalent Kinase Inhibitors

	$K_i$ ( $\mu\text{M}$ )			
	c-Src	c-Yes	Hck	c-Abl
<b>3</b> 	0.40 $\pm 0.02$	0.47 $\pm 0.02$	0.65 $\pm 0.11$	0.98 $\pm 0.16$
<b>8</b> 	0.33 $\pm 0.05$			
<b>9</b> 	0.07 $\pm 0.02$	0.61 $\pm 0.13$	0.71 $\pm 0.03$	0.73 $\pm 0.16$
<b>10</b> 	0.37 $\pm 0.02$			
<b>11</b> 	0.13 $\pm 0.06$	0.80 $\pm 0.16$	1.8 $\pm 0.1$	1.2 $\pm 0.4$
<b>EL-1061</b> 	160 $\pm 20^{\text{a,c}}$	N/A <sup>b</sup>	>1250 <sup>c</sup>	>1250 <sup>c</sup>

<sup>a</sup>Value is for 3M c-Src. <sup>b</sup>EL-1061 can bind irreversibly to c-Yes.

c-Abl, 69% similar to c-Src). While **3** has virtually no selectivity within this panel (average selectivity = 1.8 $\times$ ), both **9** and **11** have dramatically improved selectivity (average selectivity = 9.5 $\times$  and 9.4 $\times$ , respectively). Compounds selective for c-Src over c-Yes have application in pharmacological manipulation of embryonic stem cells,<sup>22</sup> and bivalent inhibitor **9** is the most selective c-Src over c-Yes inhibitor reported to date.<sup>8,23</sup> In addition, while c-Src has been validated as a target in triple-negative breast cancer (TNBC),<sup>8,24</sup> c-Abl has been reported to be a tumor suppressor in TNBC.<sup>8,25,26</sup> Thus, compounds that have selectivity for c-Src over these homologous kinases will be useful probe compounds for the biology community.

To explore the role of the non-ATP-competitive fragment in providing selectivity to bivalent inhibitor **9**, we determined the selectivity of **EL-1061** (the parent fragment of **9**) in our homologous kinase panel. **EL-1061** is a weak inhibitor of 3M c-Src ( $\text{IC}_{50}$  = 160  $\pm$  20  $\mu\text{M}$ ); however, it was found not to inhibit Hck and c-Abl at concentrations up to 1250  $\mu\text{M}$ . Because c-Yes has a P-loop cysteine we cannot determine a reversible binding constant for this enzyme. Overall, fragment **EL-1061** has a >8 $\times$  selectivity, which is consistent with the high selectivity obtained from bivalent inhibitor **9** (10 $\times$  selectivity for c-Src over Hck and c-Abl).

It is worth noting that the potency gained going from **3** to **9** was modest (6-fold) and was at the expense of ligand efficiency ( $\text{LE} = 1.4(-\log K_i)/(\# \text{ heavy atoms})$ ). ATP-competitive inhibitor **3** has a  $\text{LE} = 0.32$ , while bivalent inhibitor **9** has a  $\text{LE} = 0.24$ . Despite lowering the overall ligand efficiency, there

is a large increase in selectivity due to the addition of the non-ATP-competitive fragment. It is possible that with larger acrylamide fragment libraries (our library is 110 members), a more optimal-binding fragment could be identified.

On the basis of the selectivity data (Table 1), we propose that the non-ATP-competitive fragments are binding in the P-loop (also called the glycine-rich loop) pocket of c-Src (Supplemental Figure S2). This pocket has previously been exploited to improve the selectivity of ATP-competitive kinase inhibitors; however, all previously reported P-loop binding ligands were obtained using structure-based drug design.<sup>8,27</sup> One of the key advantages of our methodology is that no structural information is required to identify fragments capable of assembling into bivalent inhibitors.<sup>28</sup> Our hypothesis is supported by structural information for analogues of **1** in which the amide functionality would be pointing in the direction of the P-loop pocket (Supplemental Figure S3). The P-loop of c-Src has a unique extended conformation resulting from a three-residue salt bridge between Lys272, Gln275, and Glu280.<sup>29</sup> We disrupted this salt bridge by mutating Glu280 to Gly, a mutation previously shown to modulate the P-loop conformation of Lyn, a Src-family kinase.<sup>29</sup> We produced E280G c-Src and tested the ability of bivalent inhibitor **9** to inhibit this construct of c-Src. Consistent with our hypothesis, we found that **9** is a 4-fold weaker inhibitor (WT c-Src  $K_i = 0.07 \pm 0.02 \mu\text{M}$ , E280G c-Src  $K_i = 0.26 \pm 0.03 \mu\text{M}$ ). While our screen identified fragments proposed to bind in the P-loop pocket of c-Src, one could vary the linker length and/or position of the thiol to discover fragments that bind in other locations adjacent to the ATP pocket.

Herein, we have demonstrated the application of a fragment elaboration method using thiol-acrylamide chemistry to generate bivalent enzyme inhibitors. We applied our method to c-Src kinase; however, our methodology could easily be applied to other enzyme systems, including proteases and phosphatases. The assembled bivalent inhibitors possess improved potency and selectivity compared to the starting fragments. Work applying this methodology to additional kinases targeted by **1** is ongoing in our laboratory.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthetic procedures, assay details, and compound characterization data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.5b00167.

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### Author Contributions

The manuscript was written through contributions of all authors and all authors have given approval to the final version of the manuscript.

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## Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

ATP, adenosine triphosphate; FBDD, fragment-based drug discovery; TNBC, triple-negative breast cancer; WT, wild-type

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