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Substrate Activity Screening for Kinases: Discovery of Small Molecule Substrate-Competitive c-Src Inhibitors**

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Abstract

Substrate-competitive kinase inhibitors represent a promising class of kinase inhibitors, however, there is no methodology to selectively identify this type of inhibitor. Herein, we report the application of substrate activity screening to tyrosine kinases. Using this methodology, we have discovered the first small molecule substrates for any protein kinase and the first substrate-competitive inhibitors of c-Src with activity in both biochemical and cellular assays.

Characterization of our lead inhibitor demonstrates that substrate-competitive kinase inhibitors possess unique properties, including cellular efficacy that matches biochemical potency and synergy with ATP-competitive inhibitors.

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Keywords

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Protein tyrosine kinases are heavily studied targets in drug discovery.^[1–3] All FDA-approved drugs targeting tyrosine kinases inhibit kinase activity via competition with ATP. Despite the popularity and success of ATP-competitive inhibitors, there are limitations inherent to this class of kinase inhibitors. First, due to homology in the ATP binding pocket across kinases, obtaining selective inhibition for a particular kinase is exceptionally challenging.^[4] Poor selectivity leads to off-target toxicity and also limits the use of most ATP-competitive kinase inhibitors in biological experiments.^[4] Additionally, ATP-competitive inhibitors must compete with millimolar concentrations of ATP in cells, necessitating difficult to obtain affinity (pM to nM) and/or very high doses of inhibitor required to obtain potent inhibition *in vivo*.^[4] Thus, kinase inhibitors that bind outside the ATP pocket are of increasing interest.^[5–7]

Targeting the substrate-kinase interaction is challenging because this is a flat protein-protein interaction surface that lacks any obvious small molecule binding sites.^[8] Furthermore, there are no reported screening methods that can selectively identify substrate-competitive kinase inhibitors. As a result, very few substrate-competitive kinase inhibitors have been described.^[6]

Herein, we describe methodology for identifying substrate-competitive kinase inhibitors. We utilize the substrate activity screening (SAS) platform originally developed for proteases and phosphatases by Ellman and co-workers.^[9,10] SAS first identifies weak binding non-peptide substrates of an enzyme. The identified substrates are then converted into inhibitors. While there are prior reports of peptide substrates converted into peptide inhibitors for kinases, no non-peptide substrates have been reported for any protein kinase.^[6,11–14]

Our SAS method for identifying substrate-competitive kinase inhibitors consists of three steps (Scheme 1): (1) a diverse library of low molecular weight phenols is screened to identify non-peptidic kinase substrates; (2) the phenol substrates are converted into inhibitors by replacement of the phenol with non-phosphorylatable surrogates; (3) the inhibitors are optimized by analog synthesis. We developed our methodology using c-Src, a eukaryotic protein tyrosine kinase. c-Src was the first proto-oncogene described and overexpression of c-Src has been linked to progression of many cancers.^[15,16]

Step 1

A library of 88 phenols was selected using computational clustering analysis from thousands of commercially available phenols with molecular weights below 300 Da (see Supporting Information for a complete list of phenols screened). The phenol library was initially screened at 100 μ M in the presence of 1 mM ATP and 100 nM c-Src. After 30 min incubation the production of ADP (a byproduct of the kinase reaction) was measured using ADP-Glo, a luciferase-based assay.^[17]

From the phenol library, 9 non-peptidic substrates were found with >2.5% ADP formation (>2 standard deviations from the mean of the entire phenol library). Phosphorylation of substrate was verified by HPLC (see Supporting Information for details). We next obtained K_M values for the 9 substrates and found K_M values that spanned 15–522 μM (Chart 1). Significantly, these represent the only non-peptidic substrates known for any protein kinase, and 5 of the phenols have K_M values lower than an optimal pentapeptide substrate of c-Src (Ac-AIYAA-NH₂, K_M = 60 μM , Table S2).

As a measure of substrate selectivity, we determined the K_M value for each phenol against two kinases with homology to c-Src: Hck and c-Abl (85% and 70% similarity, respectively, to c-Src kinase domain). Of the 9 best c-Src phenolic substrates, four phenols were also Hck substrates while none were phosphorylated by c-Abl. This suggests that despite high sequence conservation and nearly identical ATP-binding sites, homologous kinases have divergent substrate-binding preferences, even with small molecule substrates.

Step 2

Prior work by Graves and co-workers has shown that fluorination of a substrate tyrosine yielded a peptide inhibitor for insulin receptor kinase.^[11,18] The tetrafluorotyrosine peptide bound the substrate site, however, was not phosphorylated at neutral pH.^[11,18] In addition to tetrafluorophenol, we hypothesized that pyridine N-oxide (**8**) and hydroxypyridine (**9**) might also serve as mimics of a phenol without being phosphorylatable (Chart 2).

For conversion from substrate to inhibitor, we selected p-aniline phenol **5** (K_M = 120 μM) due to its affinity and synthetic feasibility to evaluate each of the three potential phenol isosteres. Fluorination of the phenol led to inhibitor **7** with a K_i = 257 μM . Meanwhile, pyridine N-oxide (**8**) and hydroxypyridine (**9**) each provided modest inhibition (478 and 552 μM , respectively). Each inhibitor was found to be substrate-competitive in biochemical assays (Table S4) and not phosphorylated by c-Src (see Supporting Information).

We found that tetrafluorophenol **7** is selective for c-Src over Hck, while both pyridine N-oxide **8** and hydroxypyridine **9** inhibited c-Src and Hck with comparable potencies (see Supporting Information). This suggests that the optimal inhibitor pharmacophore is likely different for each kinase. While this requires additional compound synthesis, it also affords an opportunity to refine the selectivity of the inhibitor.

Step 3

On the basis of its superior potency and selectivity, we chose the tetrafluorophenol inhibitor pharmacophore for inhibitor optimization and synthesized a small focused library of tetrafluorophenol diphenyl amines (Table S3). Three members of this library had improved K_i values relative to the parent inhibitor **7** (Chart 3). The most potent analog, compound **12**, has a K_i = 16 μM and represents one of the most potent substrate-competitive kinase inhibitors reported to date,^[6] and is on par with other small molecule inhibitors of protein-protein interactions.^[19]

Inhibitor **12** is selective for c-Src over homologous kinases, which is in stark contrast to PP2, an ATP-competitive inhibitor of c-Src (Table 1).^[20,21] We tested both **12** and PP2 against the Src family kinases (9 members) and found that while PP2 had no selectivity across the family, compound **12** has an average selectivity of 6×. Within the homologous Src family, compound **12** is the most selective inhibitor for c-Src reported to date. Notably, compound **12** is >5-fold selective for c-Src over c-Yes, a Src family kinase with 95% sequence similarity and 90% sequence identity to c-Src in the kinase domain. A recent comprehensive survey of ATP-competitive kinase inhibitor selectivity found no ATP-competitive kinase inhibitors with this level of selectivity for c-Src over c-Yes.^[22] These results highlight the unprecedented selectivity that can readily be obtained with substrate-competitive kinase inhibitors.

Inhibitors identified from SAS should inherently be substrate-competitive. However, because multiple binding sites exist on protein kinases, we wanted to confirm the mode of action for inhibitor **12**. We found that the IC₅₀ was sensitive to peptide substrate concentration, but not sensitive to ATP concentration (see Supporting Information). Furthermore, Lineweaver-Burk and K_M analyses are consistent with a substrate-competitive and ATP non-competitive mode of action (see Supporting Information).

To provide further insight into the binding mode, we performed induced fit docking to flexibly dock **12** into c-Src (see Supporting Information).^[23] In the docked model, an interaction was predicted between Arg388 and inhibitor **12**. This arginine residue is replaced by an alanine in c-Abl (Arg365 in c-Abl replaces Ala390 in c-Src). We produced R388A/A390R c-Src and found that inhibitor **12** was a weak inhibitor of this enzyme (K_i = 184 μM) compared to wild-type c-Src (K_i = 16 μM). These data are consistent with the proposed binding model and, together with the biochemical analyses, strongly support a substrate-competitive mode of action.

We next evaluated compound **12** in a cellular context. In an ELISA-based assay, compound **12** was found to have an IC₅₀ = 15 μM for cellular c-Src autophosphorylation. This demonstrates both that compound **12** is cell permeable and capable of inhibiting c-Src *in cellulo*. We then tested the ability of compound **12** to inhibit the growth of SK-BR-3 and HT-29 cells in culture, cancer cell lines previously shown to be c-Src growth dependent.^[20,24] In this assay, compound **12** also has a GI₅₀ = 15 μM for SK-BR-3 and GI₅₀ = 37 μM for HT-29. Of note, compound **12** is significantly more potent than PP2 against both SK-BR-3 and HT-29 cells (Table 2).^[20] In fact, compound **12** has similar SK-BR-3 anti-proliferative activity as the most potent c-Src inhibitors known, including FDA-approved c-Src inhibitors dasatinib and bosutinib.^[25,26]

We observed excellent correlation between compound **12**'s ability to inhibit c-Src activity and the growth of a cancer cell line dependent upon c-Src activity (Table 2). In addition, compound **12** was inactive (GI₅₀ > 100 μM) against non-Src-dependent cancer cell lines MCF7 and T47D. Finally, we examined activation of c-Src dependent (Jnk and STAT3) and c-Src independent (Akt and Erk) signalling pathways in SK-BR-3 cells. We found that compound **12** inhibited activation of only Src-dependent pathways, while PP2 was active

against all four signalling pathways. Together, these data demonstrate that inhibitor **12** is acting as a highly selective c-Src inhibitor *in cellulo*.

A long-standing hypothesis of substrate-competitive kinase inhibitors posits that no significant loss in cellular potency should be observed for substrate-competitive inhibitors, because kinase substrates are present at or below their K_M .^[6] This is in stark contrast to ATP competitive inhibitors where K_M values are often low micromolar while ATP is present in millimolar concentrations.^[4,27] Inhibitor **12** represents one of very few substrate-competitive tyrosine kinase inhibitors that has activity in both biochemical and cellular assays and is the only such inhibitor of c-Src.^[6] The biochemical K_i of **12** for c-Src is nearly identical to the IC_{50} for cellular autophosphorylation of c-Src. In contrast, PP2 has a biochemical K_i = 30 nM and an IC_{50} for c-Src autophosphorylation of 2.2 μ M. Thus, while our substrate-competitive inhibitor loses no efficacy, a classic ATP-competitive inhibitor is 44-fold less active *in cellulo* (Table 2).

We also hypothesized that our substrate-competitive inhibitor could be used simultaneously with an ATP-competitive kinase inhibitor. To test this hypothesis, we used IC_{35} concentrations of compound **12** in combination with PP2 or PP5. PP2 and PP5 are well-established ATP-competitive inhibitors of c-Src that bind the active and inactive conformations, respectively.^[21,28] We found that both PP2 and PP5 were synergistic (hyper-additive) when combined with inhibitor **12** (Figure 1).^[29] Together, these data show for the first time the ability of substrate-competitive inhibitors to bind simultaneously with ATP-competitive inhibitors.

Herein, we have described the first methodology to enable discovery of small molecule substrate-competitive kinase inhibitors. This class of compounds has been proposed to have several advantages, however, a dearth of compounds prevented proper evaluation of their potential. We applied our methodology to c-Src and identified inhibitor **12** (K_i = 16 μ M). Biochemical, computational, and mutagenesis studies support a substrate-competitive mode of action. Using compound **12**, we observed nearly identical cellular efficacy compared to biochemical potency, a feature not found with ATP-competitive inhibitors. Unlike ATP-competitive inhibitors, we demonstrated that biochemical and cellular selectivity is inherent in this class of compounds. Finally, we demonstrated that substrate-competitive inhibitors can be used simultaneously with ATP-competitive inhibitors to provide synergistic inhibition of the target kinase. Our methodology is the only screening technique to selectively identify substrate-competitive kinase inhibitors and should be applicable to any tyrosine kinase of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synergy studies of combinations of substrate-competitive inhibitor **12** with ATP-competitive inhibitors PP2 or PP5. IC₃₅ concentrations are dosed individually and in combination. The dotted line denotes predicted additivity $[(eA+eB)-(eA*eB)]$ of **12** + PP2 (or PP5).^[25] A higher level of inhibition than the predicted additivity indicates synergism.

Scheme 1.
Overview of SAS methodology for protein kinases.

Chart 1.
Phenol substrates of c-Src and their corresponding K_M values.

7
 $K_i = 257 \mu\text{M}$

8
 $K_i = 478 \mu\text{M}$

9
 $K_i = 552 \mu\text{M}$

Chart 2.

Conversion to inhibitors using non-phosphorylatable surrogates of phenol.

Chart 3.
Optimized substrate-competitive c-Src inhibitors.

Table 1

Biochemical selectivity data for homologous kinases.

Kinase	12 (selectivity ratio)	PP2 (selectivity ratio)
c-Src	16 μ M	0.05 μ M
Yes	82 μ M (5)	0.05 μ M (1)
Hck	325 μ M (20)	0.09 μ M (2)
Blk	52 μ M (3)	0.07 μ M (2)
Fgr	51 μ M (3)	0.03 μ M (1)
Frk	83 μ M (5)	0.02 μ M (1)
Fyn	61 μ M (4)	0.02 μ M (1)
Lck	63 μ M (4)	0.01 μ M (1)
Lyn	60 μ M (4)	0.02 μ M (1)
c-Abl	1.0 mM (63)	0.4 μ M (9)

Table 2

Comparison of cellular data for substrate-competitive inhibitor **12** and ATP-competitive inhibitor PP2.

	12	PP2
Biochemical K_i	16 μ M	0.05 μ M
Cellular phosphorylation (IC_{50})	15 μ M	2.2 μ M
Ratio (cell/biochemical)	0.9	44