Kinase Inhibitors

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

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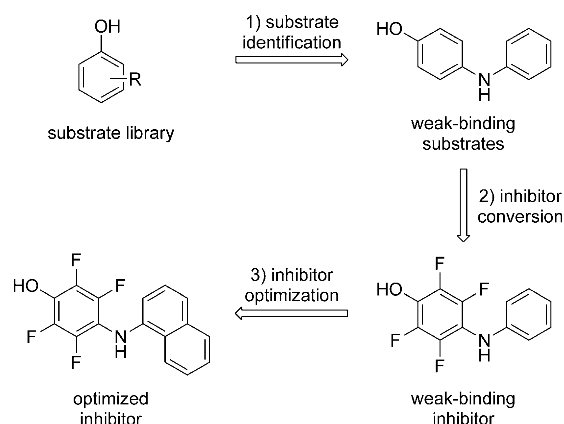
Abstract: *Substrate-competitive kinase inhibitors represent a promising class of kinase inhibitors, however, there is no methodology to selectively identify this type of inhibitor. Substrate activity screening was applied to tyrosine kinases. By using this methodology, the first small-molecule substrates for any protein kinase were discovered, as well as the first substrate-competitive inhibitors of c-Src with activity in both biochemical and cellular assays. Characterization of the lead inhibitor demonstrates that substrate-competitive kinase inhibitors possess unique properties, including cellular efficacy that matches biochemical potency and synergy with ATP-competitive inhibitors.*

Protein tyrosine kinases are heavily studied targets in drug discovery.[1–3] All FDA-approved drugs targeting tyrosine kinases inhibit kinase activity through competition with ATP. Despite the popularity and success of ATP-competitive inhibitors, there are limitations inherent to this class of kinase inhibitors. First, owing 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, thus necessitating difficult to obtain affinity (pM to nM) and/or very high doses of inhibitor to obtain potent inhibition in vivo.[4] Therefore, kinase inhibitors that bind outside of the ATP pocket are of increasing interest.[5–7]

Targeting the substrate–kinase interaction is challenging because it involves 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 a methodology for identifying substrate-competitive kinase inhibitors. We utilized 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 nonphosphorylatable surrogates; 3) the inhibitors are optimized through analogue synthesis. We developed our methodology by 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 the progression of many cancers.[15,16]



Scheme 1. Overview of the SAS methodology for protein kinases.

In the first step, a library of 88 phenols was selected by using computational clustering analysis from thousands of commercially available phenols with molecular weights below 300 Da (see the Supporting Information for a complete list of phenols screened). The phenol library was initially screened at 100 μM in the presence of 1 mM ATP and 100 nM c-Src. After 30 min incubation, the production of ADP (a byproduct

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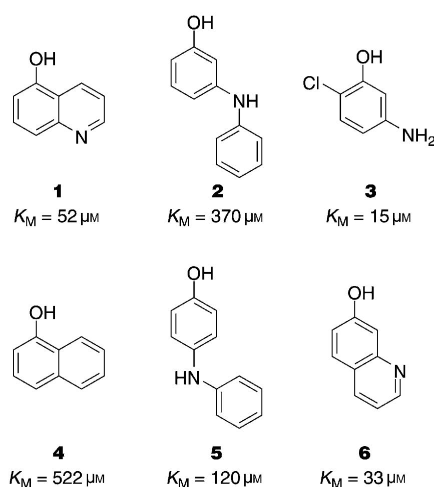
of the kinase reaction) was measured by using ADP-Glo, a luciferase-based assay.[17]

From the phenol library, nine non-peptidic substrates were found that gave more than 2.5% ADP formation (more than two standard deviations from the mean of the entire phenol library). Phosphorylation of the substrate was verified by HPLC (see the Supporting Information). We next obtained K_M values for the nine selected substrates and found K_M values that spanned 15–522 μM (Scheme 2). Significantly, these represent the only non-peptidic substrates known for any protein kinase, and five of the phenols gave K_M values lower than an optimal pentapeptide substrate for c-Src (Ac-AIYAA-NH_2, $K_M = 60$ μM; Table S2 in the Supporting Information).

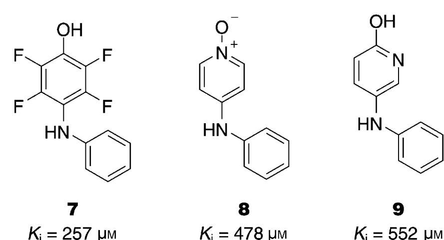


Scheme 2. Phenol substrates of c-Src and their corresponding K_M values.

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 the c-Src kinase domain). Of the nine best c-Src phenolic substrates, four phenols were also Hck substrates and 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 for small molecule substrates.

The second step built on prior work by Graves and co-workers, who showed that fluorination of a substrate tyrosine yielded a peptide inhibitor for insulin receptor kinase.[11,18] The tetrafluorotyrosine peptide bound the substrate site, however, it was not phosphorylated at neutral pH values.[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 (Scheme 3).

For conversion from substrate to inhibitor, we selected the *p*-aniline phenol **5** ($K_M = 120$ μM) because of its affinity and the synthetic feasibility of evaluating each of the three potential phenol isosteres. Fluorination of the phenol led to inhibitor **7**, which gave a K_i value of 257 μM. Meanwhile, pyridine N-oxide (**8**) and hydroxypyridine (**9**) each provided

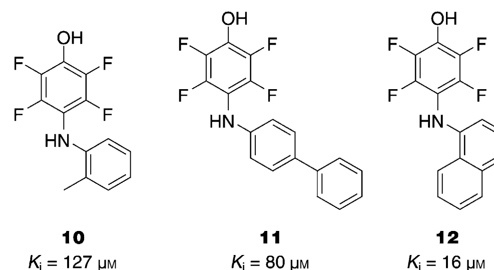


Scheme 3. Conversion into inhibitors by using nonphosphorylatable surrogates of phenol.

modest inhibition ($K_i = 478$ and 552 μM, respectively). Each inhibitor was found to be substrate-competitive in biochemical assays (Table S4) and none were phosphorylated by c-Src (see the 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 the Supporting Information). These results suggest that the optimal inhibitor pharmacophore is likely different for each kinase. While this necessitates additional compound synthesis, it also affords an opportunity to refine the selectivity of the inhibitor.

In the third step, we synthesized a small focused library of tetrafluorophenol diphenyl amines (Table S3). The tetrafluorophenol inhibitor pharmacophore was chosen for inhibitor optimization on the basis of its superior potency and selectivity. Three members of the library gave improved K_i values relative to the parent inhibitor **7** (Scheme 4). The most potent analogue, compound **12**, gave a K_i value of 16 μM and represents one of the most potent substrate-competitive kinase inhibitors reported to date,[6] on par with other small-molecule inhibitors of protein-protein interactions.[19]



Scheme 4. Optimized substrate-competitive c-Src inhibitors.

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 of kinases (nine members) and found that while PP2 showed no selectivity across the family, compound **12** showed an average selectivity of six-fold. Within the homologous Src family, compound **12** is the most selective inhibitor for c-Src reported to date. Notably, compound **12** shows more than five-fold selectivity 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

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Table 1: Biochemical selectivity data for homologous kinases.

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

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 IC_{50} values were sensitive to peptide substrate concentration but not to ATP concentration (see the Supporting Information). Furthermore, Lineweaver–Burk and K_M analyses are consistent with a substrate-competitive and ATP-noncompetitive mode of action (see the Supporting Information).

To provide further insight into the binding mode, we performed induced-fit docking to flexibly dock **12** into c-Src (see the 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 ($K_i = 184$ μм) compared to wild-type c-Src ($K_i = 16$ μм). 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 enzyme-linked immunosorbent assay (ELISA)-based assay, compound **12** was found to have an IC_{50} value of 15 μм for cellular c-Src autophosphorylation. This result demonstrates that compound **12** is both cell permeable and capable of inhibiting c-Src in cells. We then tested the ability of compound **12** to inhibit the growth of SK-BR-3 and HT-29 cells, cancer cell lines previously shown to be c-Src growth dependent.[20,24] In this assay, compound **12** produced growth inhibition with $GI_{50} = 15$ μм for SK-BR-3 and $GI_{50} = 37$ μм 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** shows SK-BR-3 antiproliferative activity similar to the most potent c-Src inhibitors known, including the FDA-approved c-Src inhibitors dasatinib and bosutinib.[25,26]

We observed excellent correlation between the ability of compound **12** 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 ($GI_{50} > 100$ μм) against the non-Src-dependent cancer cell lines MCF7 and T47D. Finally, we examined the activation of c-Src dependent (Jnk and STAT3) and c-Src independent (Akt and Erk) signaling pathways in SK-BR-3 cells. We found that compound **12** inhibited the activation only of Src-dependent pathways, while PP2 was active against all four signaling pathways. Together, these data demonstrate that inhibitor **12** acts as a highly selective c-Src inhibitor in cellulo.

Table 2: Comparison of cellular data for substrate-competitive inhibitor **12** and ATP-competitive inhibitor PP2.

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

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 in concentrations at or below their K_M values.[6] This is in stark contrast to ATP competitive inhibitors, where the 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 shows activity in both biochemical and cellular assays and is the only such inhibitor of c-Src.[6] The biochemical K_i value of **12** for c-Src is nearly identical to the IC_{50} value for cellular autophosporylation of c-Src. In contrast, PP2 has a biochemical K_i of 45 nм and an IC_{50} for c-Src autophosphorylation of 2.2 μм. 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 that enables the 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 μм). Biochemical, computational, and mutagenesis studies support a substrate-competitive mode of action. When 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

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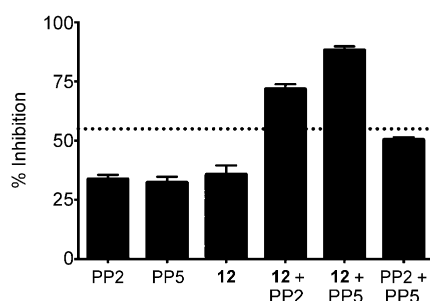

Figure 1. Synergy studies of combinations of substrate-competitive inhibitor **12** with ATP-competitive inhibitors PP2 or PP5. IC_{35} concentrations were dosed individually and in combination. The dotted line denotes predicted additivity [(eA + eB) − (eA*eB)] of **12** + PP2 (or PP5).[25] A higher level of inhibition than the predicted additivity indicates synergism.

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.

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