The pp60^{c-Src} inhibitor PP1 is non-competitive against ATP

Rotem Karni[a], Sarit Mizrachi[a], Ella Reiss-Sklan[a], Aviv Gazit[a], Oded Livnah[a,b], Alexander Levitzki[a,*]

[a]Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[b]The Wolfson Center for Structural Biology, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Abstract Glutathione-S-transferase (GST)-pp60^{c-Src} (GST-Src) expressed in *Escherichia coli* is as catalytically active as purified, activated pp60^{c-Src} protein derived from human platelets. We utilized the bacterially expressed enzyme, together with information about the structures of Src family kinases in complex with their inhibitors PP1 and PP2, to modify PP1 in a quest for improved inhibitors. Despite the detailed structural information on Hck-PP1 and Lck-PP2 complexes, which shows that PP1 and PP2 bind to the adenosine triphosphate (ATP) pocket, we were unable to improve the affinity between modified PP1 and Src. Puzzled, we examined in detail the mechanism by which PP1 inhibits the kinase activity of Src. Here we report that PP1 is non-competitive with ATP for the inhibition of Src, at variance with what is currently accepted, and is a 'mixed competitive inhibitor' vis-à-vis the substrate. These findings shed new light on the mechanism whereby PP1-like molecules inhibit Src. Examination of the homology between the kinase domain of Src and those of Hck and Lck reveals significant differences outside the ATP binding pocket, whereas they are identical within the ATP binding domain. These results suggest that PP1 may be a leading compound for ATP non-competitive inhibitors of Src family kinases. Since Src in its active form is the hallmark of numerous cancers, understanding how PP1 inhibits activated Src will aid in the discovery of potent and selective Src kinase inhibitors.
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1. Introduction

The protein tyrosine kinase pp60^{c-Src} (Src) is a major signal transduction element in both normal and transformed cells [1–3]. Src contributes significantly to the transformed phenotype of cancer cells and functions as a survival factor in cancer cells [4,5]. Moreover, we recently showed that c-Src mediates anti-apoptotic signals through regulation of the expression of the anti-apoptotic gene Bcl-X$_L$ [4]. Since Src

kinase has been found to be active in many tumors [3,6–9], Src kinase inhibitors could prove useful as anti-cancer agents for many types of cancer. We, therefore, developed a non-radioactive assay for screening activated c-Src kinase inhibitors. We show here, for the first time, that glutathione-S-transferase (GST)-c-Src, isolated from *Escherichia coli*, is as catalytically active as activated Src isolated from human platelets. It appears that the activation of GST-Src is mediated by GST-induced trans-autophosphorylation. Utilizing the three-dimensional model of PP1 crystallized with inactive Hck [10], and PP2 with the activated form of the highly homologous Src kinase family member Lck [11], we probed potential 'empty' spaces in order to fill them with substituents on PP1 or on PP2, with the aim of improving affinity. In view of the identity of the adenosine triphosphate (ATP) binding pocket among the Src family members, we expected to succeed in obtaining improved PP1 type molecules.

Our attempt to deviate from the PP1/PP2 core is important, particularly in view of the finding that PP1 is as potent against the platelet-derived growth factor receptor (PDGFR) kinase as it is against Src kinase [12]. Despite the rational design, attempts to improve on PP1/PP2 have so far met little success. We, therefore, examined the detailed kinetic mechanism by which PP1 inhibits active GST-Src. We found, surprisingly, that PP1 is non-competitive vis-à-vis ATP and is a 'mixed competitive' inhibitor vis-à-vis the substrate. These findings shed new light on the inhibitory mechanism of PP1.

2. Materials and methods

2.1. Construction of a plasmid encoding GST-Src
A blunt-ended *Nco*I fragment from plasmid pVN 1.8 encoding the neuronal c-SRC gene was ligated into the *Sma*I site of plasmid pGEX2T. The construct was transformed into *E. coli* strain DH5α.

2.2. Purification of GST-Src protein from bacteria
E. coli DH5α carrying the GST-SRC plasmid was grown to an optical density (600 nm) of 0.6–0.7, after which isopropyl-β-D-thiogalactopyranoside (IPTG) (Calbiochem) was added to 0.5 mM for 2 h. The bacteria were centrifuged at $4000 \times g$ for 10 min, and resuspended in 20 ml TEMB buffer (0.1 mM ethylenediamine tetraacetic acid (EDTA), 20 mM Tris–HCl pH 7.5, 1 mM MgCl$_2$, 1 mM β-mercaptoethanol and 1% Triton X-100) containing protease inhibitors (1.5 μM aprotinin, 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF), 2 mM benzamidine, 20 μM leupeptin). The bacteria were lysed using a high pressure French Press machine (1000 psi, twice), and the lysate was centrifuged for 15 min at $12\,000 \times g$ at 4°C. The supernatant was incubated with glutathione Sepharose beads (Pharmacia) for 30 min at 4°C. The beads were washed with phosphate-buffered saline (PBS) and the GST-Src fusion protein was eluted with

5 mM glutathione in 50 mM Tris pH 8, 1 mM MgCl$_2$. The eluted protein was kept at −70°C until use.

2.3. Src kinase assay (enzyme-linked immunosorbent assay (ELISA))

The general tyrosine kinase substrate poly(Glu–Tyr) (4:1) (PGT, Sigma) was coated onto 96-well Maxisorp plates (Nunc) by adding 125 μl 0.1 mg/ml PGT in PBS to each well. Plates were sealed and incubated for 16 h at 37°C, washed once with TBST (10 mM Tris–HCl pH 7.5, 50 mM NaCl and 0.1% Triton X-100), dried for 2–3 h at 37°C and stored at 4°C.

50 ng of purified GST-Src per well were incubated in 20 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$ with or without inhibitors for 20 min at 30°C.

Phosphorylation of tyrosyl residues was initiated by the addition of 20 μM ATP and terminated 10 min later by the addition of EDTA to a final concentration of 200 mM. Plates were washed five times with TBST and blocked with low-fat (1%) milk diluted 1:20 into TBST. For quantification of phosphorylated tyrosines, plates were incubated with rabbit polyclonal anti-phosphotyrosine serum for 1 h at room temperature, washed five times, and incubated with anti-rabbit peroxidase-conjugated antibody for 45 min.

Detection was carried out using a color reagent, 2,2′-azido-bis 3-ethylbenzihiazoline-6-sulfonic acid (ABTS) (Sigma), in citrate-phosphate buffer pH 4 containing 0.004% H$_2$O$_2$. 10 min after ABTS addition the OD at 405 nm was measured.

IC$_{50}$ values of inhibitors were determined using the REGRESSION program (Blackwell Scientific Software, Osney Mead, Oxford, UK).

2.4. Radioactive kinetic experiments

For competition with the substrate, parallel reactions were set up, with PGT at various concentrations (10, 20, 40, 80,160 μM). For each reaction, 20 ng of purified GST-Src were added to a solution containing PP1. The reactions were initiated by the addition of reaction buffer (final concentration: Tris–HCl 20 mM, 10 mM MgCl$_2$, 10 μM ATP and 3 μCi/reaction [γ-^{32}P]ATP). The reactions were incubated at 30°C for 20 min, and stopped by the addition of EDTA pH 8, 0.1 M (final concentration). Reaction samples were then absorbed onto 3 mm Whatman paper squares. The squares were washed three times for 30 min each and twice for 1 h, in 10% TCA, 1% sodium pyrophosphate, at room temperature. Squares were then washed in ethanol and dried. Radioactivity on the squares was counted by a scintillation counter using the 'Cherenkov' program. The data were analyzed by the Microsoft Excel program. IC$_{50}$ values were determined using the REGRESSION program. For competition with the substrate (ATP), 20 ng/reaction of purified GST-Src were added to a solution containing PP1 and poly(Glu–Tyr) (4:1). The reactions were initiated by the addition of reaction buffer (final concentration: Tris–HCl 20 mM, 10 mM MgCl$_2$, ATP at various concentrations (5, 10, 20, 50, 100 μM) and 3 μCi/reaction [γ-^{32}P]ATP). The rest of the assay was conducted as described above.

2.5. Src kinase inhibitors

PP1 and PP2 were synthesized as described by us previously [10].

3. Results and discussion

3.1. Production of bacterially active c-Src

We examined the phosphorylation state of GST-Src expressed in *E. coli*, and found that it was phosphorylated on both tyrosine 419, which positively stimulates Src activity, and on tyrosine 530, which negatively regulates Src activity (Fig. 1A). This phosphorylation is most probably due to the oligomerization of GST, which is known to occur (data not shown). The purified GST-Src protein was found to phosphorylate denatured enolase, as well as the general tyrosine kinase substrate poly(Glu–Tyr) (4:1). The specific activity of GST-Src, as determined by a radioactive assay, was found to be 23 nmol/min/mg, similar to the specific activity of pp60$^{c−Src}$ purified from human platelets [13].

We calibrated an ELISA assay, using the general tyrosine kinase substrate poly(Glu–Tyr) (4:1) coated on 96-well micro-titer plates [14–17] (Fig. 1B, and data not shown). The K_m of GST-Src for ATP (Fig. 1C) is 8.55 μM, which is lower than that of recombinant c-Src protein from baculovirus, reported to be 30 μM [16] or 37 μM [18].

After calibration of the assay, we tested the specificity of the system. Known Src and other tyrosine kinase inhibitors were examined for their abilities to inhibit GST-Src. As expected, the known Src kinase inhibitors, PP1 and PP2 [19], potently inhibited GST-Src activity, with IC$_{50}$ values of 50 (for PP1) and 100 nM (for PP2) (Table 1). Other tyrosine kinase inhibitors had far lower inhibitory effects on GST-Src activity than on their target kinases (Table 2).

3.2. The efficacy of PP1/PP2 analogs designed on the basis of the three-dimensional structure of Src

According to the solved structure of Src family kinases, in complex with inhibitor PP1 [10] or PP2 [11], the binding site of these inhibitors is located in the crevice between the N- and C-lobes of the Src kinase domain (Fig. 2, and [10]). There are several structures of Hck in complex with inhibitors available in the Protein Database (PDB). These structures are of the inactive, unphosphorylated form of the kinase domain, and thus the two lobes are in the closed conformation. The structures include complexes with adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) (PDB code 1AD5), quercetin (PDB code 2HCK) [20] and PP1 (PDB code 1QCF) [10]. These structures reveal that the pyrazolopyrimidine rings of AMP-PNP and of PP1 in the complexes are positioned in similar orientations at the binding site. The tuloyl group is thus located inward from the binding pocket and the phosphate moiety of AMP-PNP and t-butyl in PP1 is positioned towards the portal area of the binding site. Recently, structures of the phosphorylated, open form of the Lck kinase domain complexed with AMP-PNP, staurosporine and PP2 (PDB codes 1QPC, 1QPD, 1QPE respectively) were reported [11]. The structures of activated Lck in complex with AMP-PNP and PP2 demonstrate, as in the case of Hck, that the pyrazolopyrimidine ring systems of the inhibitors are in similar orientations. Superimposition of the Lck-AMP-PNP and Hck-AMP-PNP complexes reveals that the pyrazolopyrimidine ring systems and the sugar moieties are in similar orientations at the binding sites, whereas the phospho chains adopt somewhat different conformations in the two structures. Moreover, in the complex of Lck with AMP-PNP, there is a 1,2-ethanediol molecule at the binding site, which is not present in the Hck-AMP-PNP structure. Based on structural analysis of the Lck-PP2 complex, we can identify cavities in the binding pocket that could theoretically accommodate additional structural elements attached to the two-ring system. Examination of the homology between Src and Hck/Lck reveals that the amino acid residues which bind APPNHP are identical (fig. 6 in [10]). We thus reasoned that it was legitimate to test PP1 and its analogs on active Src.

18 rationally designed compounds were synthesized (Gazit and Levitzki, unpublished) and examined for inhibition of GST-Src (data not shown). Like PP1 and PP2, all of these compounds contain the pyrazolopyrimidine structural element, which is positioned in a consensus conformation in both the closed (Hck-PP1, AMP-PNP) and open (Lck-PP2, AMP-PNP) conformations of the kinase domain. Looking at the three-dimensional structures, we probed the 'empty' space near the toluyl group in PP1. We tried to 'fill' the space by elongation of this substituting group. Contrary to our expect-

A

97.4 KDa —

66 KDa —

P-Y   p-Y-416   p-Y-529

← GST-Src

B

◆ 160 ng GST-Src
■ 50 ng GST-Src

OD (405 nm) vs Time (min)

C

Michaelis–Menten enzyme kinetics
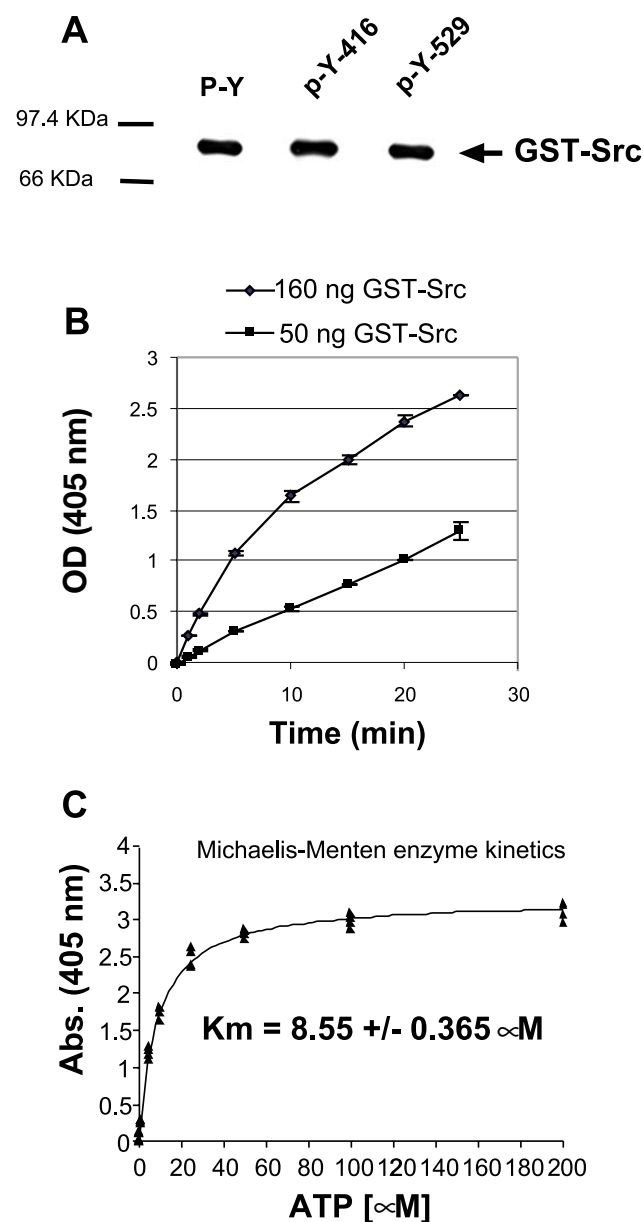
Abs. (405 nm) vs ATP [∝M]

Km = 8.55 +/− 0.365 ∝M

Fig. 1. Properties of bacterial GST-Src-linked immunosorbent assay. A: Purified GST-Src was run on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and after Western blotting, the membrane was probed with anti-phosphotyrosine (P-Y-20, 1:2000, Santa Cruz), anti-phospho-Y-419 (1:1000, Biosource), and anti-phospho-Y-530 (1:1000, Biosource) antibodies, as depicted. B: Calibration of reaction time using 0.1 mg/ml PGT-coated plate and 4 μg GST-Src/ml. C: Michaelis–Menten kinetic plot and the K_m value of ATP for GST-Src as was measured by the ELISA assay and calculated using the REGRESSION program (Blackwell Scientific Software, Osney Mead, Oxford, UK).
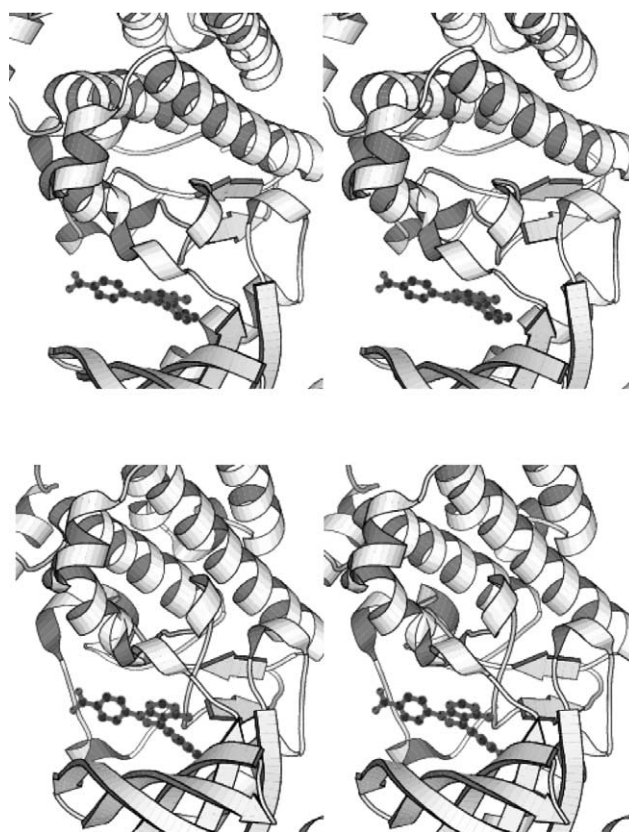

Fig. 2. A model stereoview of compound 16 in the Lck site. The compound 1-(4-carboxyphenyl)-3-(4-methylphenyl)-4-amino pyrazolo [3.4-d] pyrimidine was synthesized and modeled into the PP2 binding site based on the Lck-PP2 complex (1QPE). The structure demonstrates that the benzoic acid moiety is positioned in the portal area of the binding site and there is no apparent steric restriction. The low affinity ($IC_{50} = 1000$ μM, data not shown) could not be correlated with the structure. The figure was prepared using MOLSCRIPT program.

ations, we found no compound with better efficacy than PP1 or PP2 (data not shown). Similarly, attempts to improve the affinity of the inhibitor by replacing the t-butyl group also failed (data not shown). From analysis of the results vis-à-vis the three-dimensional structure, it is impossible to correlate the experimental IC_{50} values (data not shown) on Src with the structural information available for Hck-PP1 and Lck-PP2. This is quite puzzling since the molecules, when modeled (Fig. 2), occupy the binding site without any significant steric hindrance. In Fig. 2 we modeled the compound 1-(4-carboxyphenyl)-3-(4-methylphenyl)-4-amino pyrazolo [3.4-d] pyrimidine, which we prepared according to the spaces 'available', based on the structure of Lck-PP2. This compound was expected to possess very good affinity for c-Src,

Table 1
Activity of other selective tyrphostins against GST-Src

Inhibitor	Target	GST-Src IC$_{50}$ (μM) (*cell-free*)	Target IC$_{50}$ (μM) (*cell-free*)
PP1	Src kinase	0.05	0.05
PP2	Src kinase	0.1	0.1
AG 490	Jak-2	17	–
AG 538	IGF-1R	2	0.06
AG 825	HER-2	>100	0.35
AG 1295	PDGFR	>50	0.4
AG 1478	EGFR	0.5	0.005

Table 2
The inhibition of PTKs and Ser/Thr kinases by PP1 and PP2

	GST-Src	EGFR	PDGFR	PKA	PKB
PP1	50	600	> 50 000	> 50 000	> 50 000
PP2	100	ND	> 50 000	> 50 000	> 50 000

The inhibition of various kinases by PP1 and PP2 is described. The assays were conducted as described in Section 2 and as described in [31] (for EGFR), [28] (for PDGFR), and [32] (for PKA and PKB).

but in practice was found to be a poor binder ($IC_{50} = 1000$ nM). Similar findings were obtained for 18 compounds with various substitutions. None of these improved affinities over PP1 or PP2. These unexpected findings convinced us that the mode of binding of PP1 within the active site of active Src in solution is different from its binding to Hck or from the binding of PP2 to Lck within the crystal structure, although these three enzymes are identical in their APPNHP binding sites. Furthermore, the amino acids involved in PP1 binding in Hck are identical to those present in the $pp60^{c-Src}$ and Lck ATP binding sites (Table 4). Since, in the original publication by Hanke et al., it was claimed that 'Preliminary studies with PP1 (data not shown) show complex kinetics for inhibition of Lck...', it is likely that the inhibition of Lck by PP1 is not ATP competitive. Our findings, together with this statement, prompted us to examine in detail the kinetics of inhibition of GST-Src by PP1.

3.3. PP1 and PP2 are not ATP competitors and are 'mixed competitive' vis-à-vis the substrate

PP1 and PP2 did not compete with ATP, while the ATP mimic AMP-PNP was competitive with ATP in the same assays (Table 3, Fig. 3). In the ELISA assay and in the radioactive assay, increasing the concentration of ATP did not affect the IC_{50} values for PP1 and PP2, whereas it elevated the IC_{50} values of AMP-PNP (Table 3 and Fig. 3A, C). PP1 was also found to be 'mixed competitive' with the substrate (Fig. 3B). The data show that PP1 decreases the rate constant for product formation, but at the same time increases the affinity of the enzyme for the substrate [21]. These results demonstrate that PP1 does not compete with ATP for binding to active Src, and at the same time is a 'mixed competitive' inhibitor with regard to the Src substrate poly(Glu–Tyr) (4:1). These results suggest that PP1 and PP2 do not bind to active Src in the same manner as they bind to Hck and Lck, respectively. PP1 and PP2 seem to inhibit Src by binding to an area of the molecule that does not overlap with the ATP binding domain. The binding of the inhibitor, however, interferes with
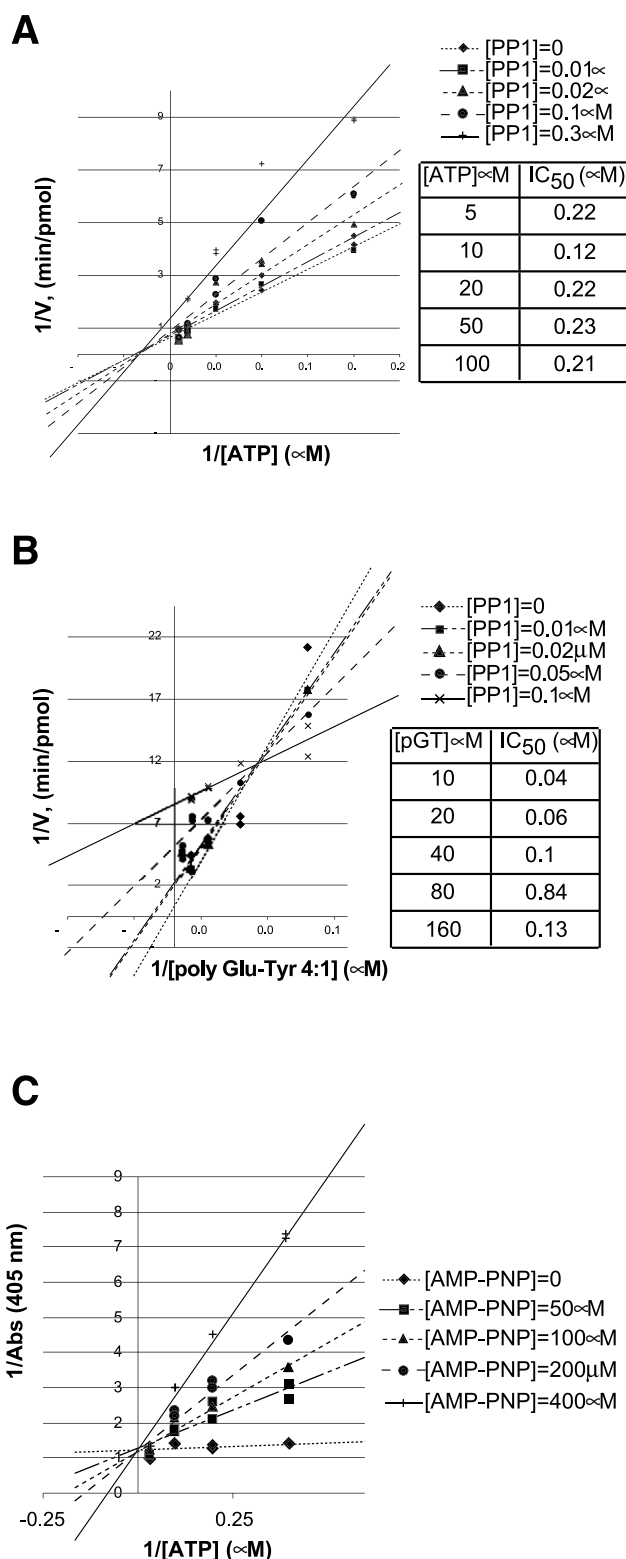
→

Fig. 3. Inhibition of Src by PP1/PP2 is non-competitive with ATP and 'mixed competitive' with substrate. A: Lineweaver–Burk plots of a radioactive assay of GST-Src. The activities of Src were determined using the radioactive assay, as described in Section 2. The reactions were performed in the presence of poly(Glu–Tyr) (4:1), at a fixed concentration, with (80 μM), and varying concentrations of ATP (5, 10, 20, 50, 100 μM). Each point represents the average of duplicate values. This is a plot of one of three experiments, all within less than 5% of each other. B: Lineweaver–Burk plots of a radioactive assay of GST-Src in the presence of ATP (10 μM), at a fixed concentration, and varying concentrations of poly(Glu–Tyr) (4:1) (10, 20, 40, 80, 160 μM). C: Lineweaver–Burk plots of an ELISA assay of GST-Src, as described in Section 2. The reactions were performed in the presence of Glu–Tyr (4:1), at a fixed concentration, (coated on the plates), and varying concentrations of ATP (2.5, 5, 10, 30, 100 μM) or AMP-PNP.

the binding of the substrate, since it is 'mixed competitive' (Fig. 3).

3.4. Structure activity considerations for future Src inhibitors

Over the past decade, several classes of potent tyrosine kinase inhibitors that bind to the ATP site ('ATP mimics') have been discovered (for review see [22]). This is in addition to

A:

[ATP] μM	IC_{50} (μM)
5	0.22
10	0.12
20	0.22
50	0.23
100	0.21

B:

[pGT] μM	IC_{50} (μM)
10	0.04
20	0.06
40	0.1
80	0.84
160	0.13

Table 3
IC$_{50}$ values of PP1, PP2, and AMP-PNP at different ATP concentrations

[ATP] (μM)	IC$_{50}$ of PP1 (μM)	IC$_{50}$ of PP2 (μM)	IC$_{50}$ of AMP-PNP (μM)
2.5	0.065	–	56.7
5	0.047	–	128.2
10	0.054	0.066	176.3
30	0.065	0.055	505.1
100		0.066	957

IC$_{50}$ values were calculated from the ELISA assay for GST-Src as described in Section 2.

inhibitors that are substrate competitive [23,24] and inhibitors that compete with both substrate and ATP, namely bi-substrate inhibitors [25,26]. The ATP mimics are hetero-bicyclic or hetero-tricyclic compounds whose core structure bears close resemblance to adenine. Not surprisingly, they all bind at the ATP sites of the tyrosine kinases. The resemblance to adenine is especially evident in the pyrrolo or pyrazolo pyrimidine family of Src kinase inhibitors and the quinazoline inhibitors of the epidermal growth factor receptor (EGFR) [27], which possess 1,4-diaza and 4-amino or anilide hetero substitution patterns that are identical or very similar to adenine. The quinoxaline inhibitors of the PDGFR possess a 1,3-diaza substitution [28] but lack the 4-amino moiety, and thus are less similar to adenine. Indeed, we have previously shown that bicyclic quinoxalines are 'mixed competitive' inhibitors of activated PDGFRβ vis-à-vis ATP [29]. Interestingly, all these inhibitors possess, in addition to the core pharmacophore, an aryl group, which interacts with a lipophilic pocket near the ATP binding site. This extra binding seems to be essential for the potency of the inhibitors and also seems to be the main factor in their selectivity. This is exemplified by the aryl at position 3 in the quinazolines, which are selective for PDGFR [28], and at position 4 in quinoxalines, which are selective for EGFR [27]. Thus, small differences in the very highly homologous ATP binding sites of tyrosine kinases have made it possible to identify selective inhibitors, although a decade ago many thought this unlikely [30]. All these compounds were discovered mainly through random screening, guided by the simple rationale that aromatic systems are likely to substitute for ATP. The recent X-ray studies of Src kinase family proteins co-crystallized with inhibitors – Hck in complex with PP1 [10] and Lck with PP2 [11] – revealed the exact binding mode for these inhibitors in the ATP pocket in the crystal.

It has also been shown that small changes in ATP competitive inhibitors (like 4-anilinoquinazolines) can modify their kinetic characteristics leading to dual-site inhibitors that are competitive with both ATP and substrate [26]. Such experiments demonstrate the proximity of these two subsites. The relative selectivity of PP1/PP2 towards active Src may be accounted for by their binding to a unique domain close to the substrate site but removed from the ATP binding domain. It is, therefore, important to co-crystallize active c-Src with PP1 or PP2 and compare the structure with that of Hck-PP1 and Lck-PP2. We believe that a substrate competitive inhibitor or a substrate 'mixed competitive' inhibitor may be useful for generating an inhibitor with strong selectivity for Src. For therapeutic use, it may be necessary to inhibit Src, but not Lck or Hck, which have pivotal roles in signaling hematopoietic immune cells. The sequences of Src, Lck and Hck at domains adjacent to the ATP binding sequences show significant differences (Table 4). It is therefore possible that PP1 actually binds to the ATP-adjacent domain. This would account for the significant differences in the IC$_{50}$ values for PP1 for Hck (20 nM), Lck (5 nM), Fyn (6 nM) and Src (170 nM), as reported by Hanke et al. [19]. These differences may eventually be exploited to generate selective Src kinase inhibitors.

Table 4
Comparison of the kinase domains of c-Src, Lck and Hck


```
c-Src(318) RHEKLVQLYAVVSEEPIYIVTEYMSKGSLLDFLKGETGKYL
Lck (296) QHQRLVRLYAVVTQEPIYIITEYMENGSLVDFLKTPSGIKL
Hck (318) QHDKLVKLHAVVTKEPIYIITEFMAKGSLLDFLKSDEGSKQ
                            ▲
                         Thr 338
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Sequences for part of the kinase domains of c-Src, Lck and Hck. The red letters represent the unique amino acids in c-Src. Thr 338 represents the conserved residue in the Src ATP binding domain [10]. The bar represents the sequence involved in the binding of ATP.

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