Letters

Fragment Screening and Assembly: A Highly Efficient Approach to a Selective and Cell Active Protein Tyrosine Phosphatase 1B Inhibitor

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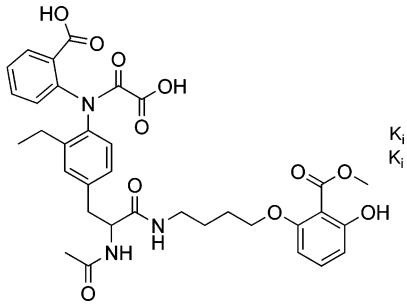
Abstract: Using an NMR-based fragment screening and X-ray crystal structure-based assembly, starting with millimolar ligands for both the catalytic site and the second phosphotyrosine binding site, we have identified a small-molecule inhibitor of protein tyrosine phosphatase 1B with low micromolar inhibition constant, high selectivity (30-fold) over the highly homologous T-cell protein tyrosine phosphatase, and good cellular activity in COS-7 cells.

Protein tyrosine phosphatase 1B (PTP1B), an intracellular protein tyrosine phosphatase (PTPase), has been implicated in negative regulation of insulin and leptin signal transduction pathways among other biological functions.[1] Mice lacking functional PTP1B exhibit the phenotypes of increased insulin sensitivity, improved glucose tolerance, and resistance to diet-induced obesity.[2,3] A synthetic small molecule that selectively inhibits PTP1B action is therefore expected to have similar beneficial effect in human and might be developed into a therapeutic for the treatment of type II diabetes and obesity.

PTPases are highly specific for the doubly charged phosphotyrosine (pTyr) residue. The crystal structures of over a dozen PTPases (mostly catalytic domains) have been determined.[4] Besides the highly conserved key structural features, it was noted that the PTP1B catalytic site and vicinal binding sites are mostly surface-exposed, highly hydrophilic, and homologous to other PTPases.[5,6] As a result, known potent PTP1B inhibitors tend to be large (MW > 500), highly charged, and cell-impermeable, restricting the ability of the inhibitors to reach the intracellular location of PTP1B.[7,8] Several less charged PTP1B inhibitors have been reported, although their exact mechanisms of action are questionable.[9]



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K_i = 0.018 ± 0.004 μM (PTP1B)
K_i = 0.065 ± 0.015 μM (TCPTP)

Figure 1. Previously reported oxamic acid based selective PTP1B inhibitor **1**.

We envisioned that selective and cell-permeable PTP1B inhibitors interacting with the catalytic site must therefore possess smaller molecular size and no more than one carboxylic acid. Targeting a second phosphotyrosine binding pocket (site 2) near the catalytic site has been proposed as one possible strategy for gaining additional potency and selectivity.[10] Such strategy has been validated with the identification of potent and selective, yet highly charged PTP1B inhibitors from this laboratory.[11,12] We now report the discovery of a moderately potent, highly selective, and cellular active PTP1B inhibitor using an NMR-based fragment screening[13] and an X-ray crystal structure based assembly approach. This approach offers a highly efficient alternative to traditional high-throughput screening (HTS) for identifying novel ligands that preferentially bind to PTP1B and possess favorable physiochemical properties.

The general idea of this approach has been illustrated by the discovery of oxamic acid based PTP1B inhibitors.[11] While potent, these highly charged inhibitors were not cell-permeable. The lack of cellular permeability and activity by this series of inhibitors had to be circumvented through an ester prodrug approach, as exemplified by inhibitor **1** (Figure 1).[14] To ensure improved cell permeability of a new generation of PTP1B inhibitors without resorting to a prodrug approach, we decided to screen only monocarboxylic acid or non-carboxylic acid-based fragments for potential catalytic site ligands. Since *N*-phenyloxamic acid appears to be the most potent non-phosphorus-containing pTyr mimetic,[15] we designed a series of heterocycle carboxylic acids as potential *N*-phenyloxamic acid mimetics with reduced pK_a. Some of them (**2**−**5**) are shown in Figure 2. These compounds, when tested at 300 μM, were found to be inactive in a colorimetry-based PTP1B-mediated *p*-nitrophenyl phosphate (*p*NPP) hydrolysis assay. The inhibition constants (K_i values) of these compounds were estimated to be greater than 900 μM by extrapolation of the Michaelis−Menten plots.

These compounds were also subject to the more sensitive NMR-based screening for possible interaction with the catalytic site. Human PTP1B (residues 1−288)

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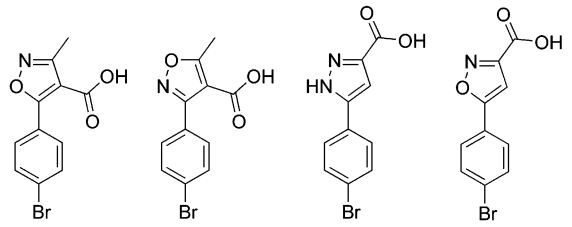


Figure 2. Selected heterocycle carboxylic acids designed to mimic *N*-phenyloxamic acid as potential pTyr mimetics.

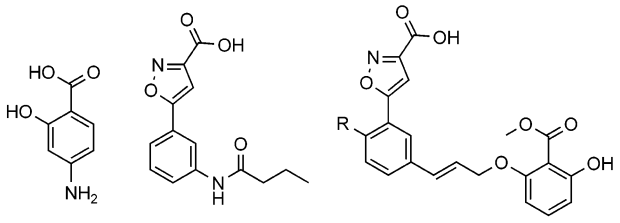


Figure 3. Site 2 ligand and PTP1B inhibitors discovered by fragment screening and assembly approach.

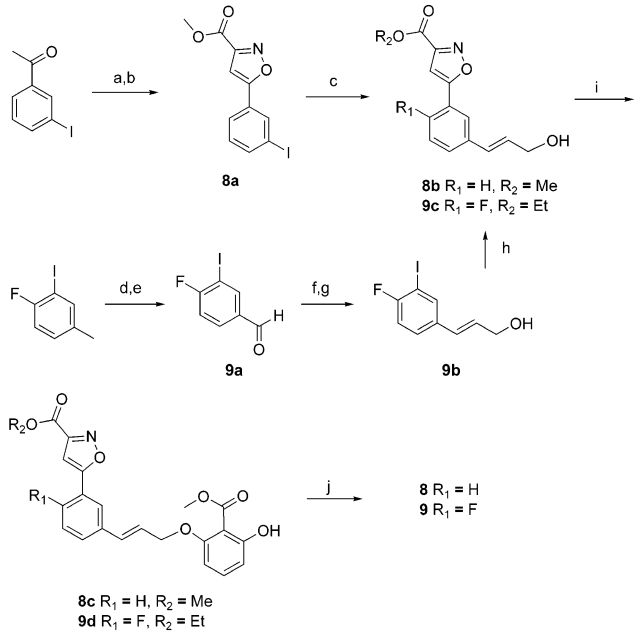
selectively labeled with ^{13}C at the methyl groups of isoleucine residues ($\delta 1$ only) was used,[16] and isoxazole carboxylic acid **5** (Figure 2) was identified as a weak binder with a dissociation constant (K_d) of 800 μM through monitoring of the chemical shift change of catalytic site residue Ile219.

Previously, salicylic acid **6** (Figure 3) has been identified as a site 2 ligand ($K_d = 1.2$ mM) through an NMR-based screening using PTP1B selectively ^{13}C-labeled at the methyl groups of methionine residues. Structure-based linking with a catalytic site directed oxamic acid based pharmacophore led to the identification of potent PTP1B inhibitor **1** with 4-fold selectivity over the highly homologous T-cell PTPase (TCPTP).[14] The binding mode of the neutral methyl salicylate based site 2 ligand was established through X-ray crystallography.

To link the catalytic site ligand **5** and site 2 methyl salicylate ligand effectively, the binding mode of catalytic site ligand **5** in PTP1B needs to be determined. However, the crystal structure of **5** complexed with PTP1B could not be solved because of its weak binding affinity. Fortunately, a related analogue **7** (Figure 3) was potent enough against PTP1B ($K_i = 148$ μM) for X-ray crystal structure determination of the complex. The X-ray structure of **7** bound to PTP1B (data not shown) suggested that a four-atom linker off the meta position of the phenyl ring should be optimal for linking with the methyl salicylate based site 2 ligand.

The orientation of methyl salicylate in site 2 was modeled according to the X-ray crystal structure of **1**.[14] Similar to inhibitor **1**, linking catalytic site binder **5** to the 6-position of methyl salicylate through an ether linkage was determined to be the most effective way for assembly. Therefore, a C_2 symmetrical methyl 2,6-dihydroxybenzoate was used for linking. Improvement of the initially linked molecules by conformational modification of the linker led to the identification of inhibitor **8** (Figure 3), which showed a K_i of 5.7 $\pm$ 0.9 μM against PTP1B. Electron-withdrawing groups were then introduced into the central phenyl ring to maximize the π-stacking interaction with Tyr46. Among the analogues examined, an equally potent inhibitor **9** (Figure 3, $K_i = 6.9 \pm 2.3$ μM) was identified by fluorinating the 6-position of the phenyl ring of **8**. **8** and **9** represent a new class of monocarboxylic acid based PTP1B inhibitors.

Scheme 1. Preparation of PTP1B Inhibitors **8** and **9**[a]



[a] Reagents and conditions: (a) dimethyl oxalate, NaOMe, MeOH, room temp; (b) $NH_2OH \cdot HCl$, *p*-TsOH, MeOH, room temp; (c) 3-tributylstannyl prop-2-en-1-ol, $Pd_2(dba)_3$, CuI, $P(2\text{-furyl})_3$, DMF, room temp; (d) NBS, BPO, CCl_4, 70 °C; (e) DMSO, solid $NaHCO_3$, 120 °C; (f) $(EtO)_2POCH_2CO_2Et$, NaH, THF, room temp; (g) DIBAL-H, CH_2Cl_2, 0 °C to room temp; (h) 5-tributylstannyl-isoxazole-3-carboxylic acid ethyl ester, $Pd_2(dba)_3$, CuI, $P(2\text{-furyl})_3$, DMF, room temp; (i) methyl 2,6-dihydroxybenzoate, DEAD, Ph_3P, THF, room temp; (j) NaOH, THF/MeOH, room temp.

Both **8** and **9** demonstrated greater than 30-fold selectivity (K_i of 202 $\pm$ 26 and 164 $\pm$ 1 μM, respectively) over TCPTP,[17] the most homologous PTPase, and showed no inhibitory activity against LAR, CD45, cdc25, and SHP-2 at the highest concentration tested (300 μM). Interestingly, for the first time, inhibitor **7**, which interacts only with catalytic site, also showed a smaller degree of selectivity (4-fold) against the highly homologous TCPTP ($K_i = 634$ μM). The exact origin of such selectivity is unclear, although the minor amino acid residue differences in the catalytic domain between PTP1B and TCPTP[18] could affect the intrinsic recognition capability for different substrates and inhibitors. From the structure−activity relationship of these compounds, site 2 binding moiety contributes about 10-fold selectivity over TCPTP.[19] To the best of our knowledge, **8** and **9** represent the most selective PTP1B inhibitors over TCPTP reported to date.[20]

The synthesis of **8** and **9** is outlined in Scheme 1. Briefly, Stille coupling[21] between the readily available isoxazole ester **8a** and tributylstannylpropenol yielded the allyl alcohol **8b**. Subsequent Mitsunobu reaction with methyl 2,6-dihydroxybenzoate and saponification of the resultant ester **8c** yielded inhibitor **8**. Similarly, Horner−Emmons olefination of aldehyde **9a** with phosphonoacetate provided a cinnamate, which was then reduced to allyl alcohol **9b**. Stille coupling of iodide

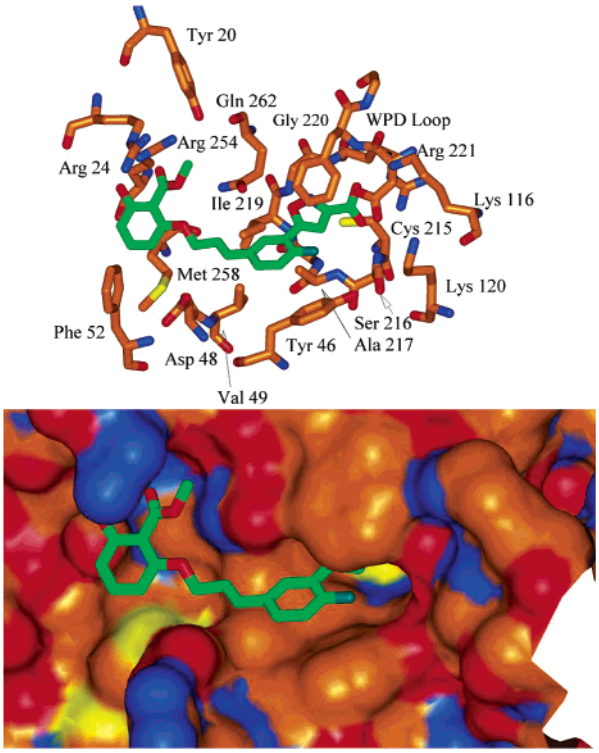


Figure 4. X-ray crystal structure of PTP1B complexed with **9**. Carbon is in orange and green for **9**, oxygen is in red, nitrogen is in blue, sulfur is in yellow, and fluorine is in dark-green.

9b with tributylstannylisoxazole yielded **9c**. Following the standard Mitsunobu and hydrolysis sequence, ester **9d** was converted to the desired inhibitor **9**.

The X-ray crystal structure of PTP1B (residues 1−322)/**9** complex was solved at a resolution of 2.6 Å, which is shown in Figure 4.[22] The isoxazole carboxylic acid binds to the active site of PTP1B with the WPD loop in the closed conformation. Bidentate-type hydrogen bonds between carboxylic acid and Arg221 provided the critical interactions. The isoxazole and phenyl rings sit snugly in the hydrophobic pocket normally occupied by the phenyl ring of pTyr. The hydrogen bond network between **9** and PTP1B remained essentially the same as that between **1** and PTP1B. The ether oxygen of **9** hydrogen-bonds with Gln262. The salicylate carboxylate group adapts an out-of-plane conformation and is in hydrogen-bond contact with Arg254. The hydroxyl group of **9**, which should remain as a neutral group at physiological pH (∼7.4), hydrogen-bonds with Arg24 and Arg254. The aromatic portion of the salicylate lies on top of the hydrophobic side chain of Met258, providing complementary van der Waals interaction.

Leptin causes tyrosine phosphorylation of the signal transducer and activator of transcription 3 (STAT3) via activation of the Janus kinase 2 (JAK2). PTP1B has been shown to negatively regulate leptin pathways by dephosphorylating JAK2 both in vitro[23] and in vivo.[24] **9** was analyzed in a COS-7 cellular system for its ability to reverse PTP1B-dependent inhibition of STAT3 phosphorylation. Initially, **9** was found to enhance the steady-state phosphorylation of STAT3 in COS-7 cells in a dose-dependent manner (10−100 μM) through a mechanism of inhibiting the endogenous level of JAK2-regulating PTPases, including PTP1B (Figure 5).

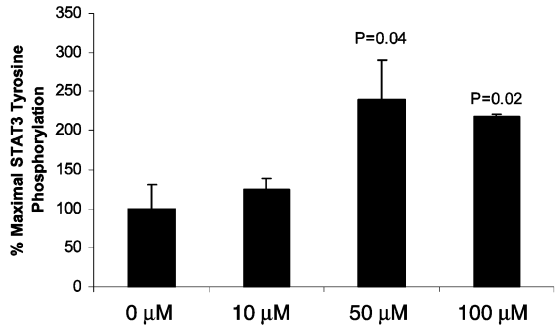


Figure 5. Reversal of dephosphorylation of STAT3 in COS-7 cells by PTP1B inhibitor **9**.

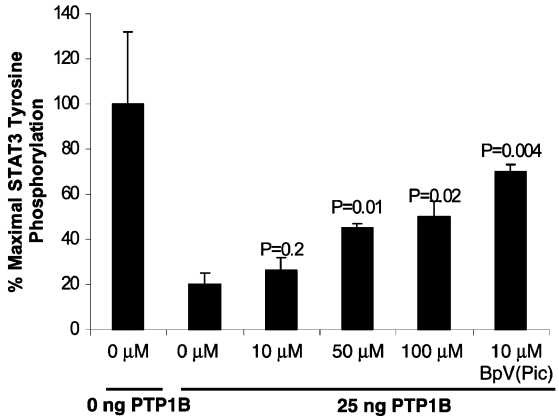


Figure 6. Reversal of PTP1B-dependent dephosphorylation of STAT3 in COS-7 cells by PTP1B inhibitor **9**.

In a more mechanism-specific way of assaying cellular PTP1B inhibition by **9**, COS-7 cells were transiently transfected with exogenous PTP1B. The inhibition of STAT3 phosphorylation caused by the overexpression of PTP1B was partially reversed by **9** dose-dependently (10−100 μM), similar to the effect exerted by a nonspecific phosphatase inhibitor BpV(pic)[25] at 10 μM (Figure 6). Consistent with the cellular activity, **9** showed moderate cellular permeability ($P_{app} > 1 \times 10^{-6}$ cm/s) in a Caco-2 cell membrane assay.[26] Interestingly, **8** showed low Caco-2 permeability ($P_{app} < 1 \times 10^{-6}$ cm/s) and much reduced cellular activity in COS-7 cells (data not shown). This beneficial fluorine effect originated most likely from the increased lipophilicity of **9**.[27] In the literature, there are only two examples of PTP1B inhibitors with cellular activity without resorting to a prodrug approach.[28,29] However, in both accounts, no selectivity data against TCPTP were reported on those peptidomimetic PTP1B inhibitors.

In conclusion, the extremely sensitive NMR-based screening allowed the identification of weak ligands for both the catalytic site and a second phosphotyrosine binding site. Structure-based assembly of these simple ligands led to the efficient discovery of moderately potent, highly selective, and cellular active PTP1B inhibitor **9**. Apart from the general applicability in the early stages of drug development, an advantage of this approach is that it starts from screening a small number of compounds with predefined properties. The identified ligands thus allow for expansion to generate molecules with optimized affinity, selectivity, and cellular activity, minimizing the immediate risk of obtaining lead molecules with disadvantageous pharmacokinetic proper-

ties. Therefore, structure-based fragment screening and assembly provide valuable tools for quick identification of novel ligands with specific binding properties, and the results compared favorably to the hits from HTS.

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Supporting Information Available: Experimental details and analytical and spectral data of key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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