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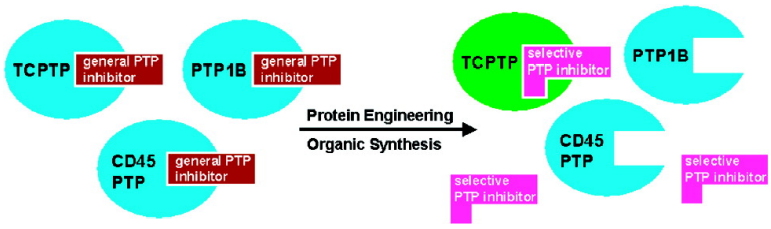

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Allele-Specific Inhibitors of Protein Tyrosine Phosphatases

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Small molecule ligands that specifically control the function of each cellular protein would represent important tools for elucidating basic biology and for validating putative therapeutic targets. General methods for systematically identifying target-specific ligands for members of large protein families are necessary prerequisites toward this objective. The engineering of protein/small molecule interfaces is now well established as a means of generating bioactive molecules with high target specificity.[1] By introducing chemical diversity into the target protein (through mutagenesis) as well as a small molecule (through organic synthesis), novel and specific ligand/receptor pairs can be efficiently designed.[1] Protein tyrosine phosphatases (PTPs),[2] a superfamily of over 100 human enzymes, catalyze the dephosphorylation of phosphotyrosine, a critical post-translational control element in eukaryotic signal transduction. Due to the large size of the PTP superfamily and the conservation of PTP active sites, the identification of inhibitors that are specific for each wild-type PTP is not a practical prospect. Here, we describe a general method for the design of selective inhibitors of engineered PTPs through rational redesign of a PTP/inhibitor interface.

To engineer unnatural inhibitor sensitivity into the PTP family, we selected a valine residue (Val49) and an isoleucine residue (Ile219) from human PTP1B as candidate inhibitor sensitization sites (Figure 1A,B). PTP1B was chosen as a "prototype" PTP; hydrophobic, aliphatic residues at positions 49 and 219 are strongly conserved across the family of classical PTPs, suggesting that other PTPs may also be susceptible to an inhibitor sensitization strategy that targets these residues (Figure 1B).[2] To generate PTP1B variants with unnatural inhibitor sensitivity, we expanded the enzyme's active site via introduction of glycine and alanine mutations at positions 49 and 219. Glycine mutations, as well as double alanine mutations, significantly compromised the catalytic activity of PTP1B, and these mutants were not investigated further (Table 1). However, the single alanine mutants, V49A and I219A PTP1B, dephosphorylated the artificial substrate, *para*-nitrophenyl phosphate (*p*NPP), and the phosphopeptide, DADEpYLIPQQG (DAD), with turnover numbers comparable to those of wild-type PTP1B (Table 1), leading us to surmise that these mutant enzymes may be suitable for an inhibitor design strategy that requires a functionally silent mutation.[5]

In an attempt to identify small molecule inhibitors that are selective for the PTP1B mutants, we synthesized a series of derivatized compounds based on a previously described, nonselective PTP inhibitor, compound **1** (Figure 1C).[3] The crystal structure of **1** bound to PTP1B has been reported; **1** binds in the PTP1B active site, using its oxalic acid functionality to construct a hydrogen-bonding array that mimics the phosphate group of phosphotyrosine (Figure 1A).[3] We used the indole nitrogen of **1**, which binds in a position that is close in space to Val49 and Ile219, as a "hook" onto which chemical groups could be synthetically appended. These bulky groups were designed to complement our space-creating mutations at positions 49 and 219, potentially leading to favorable binding interactions that are only possible in a mutated

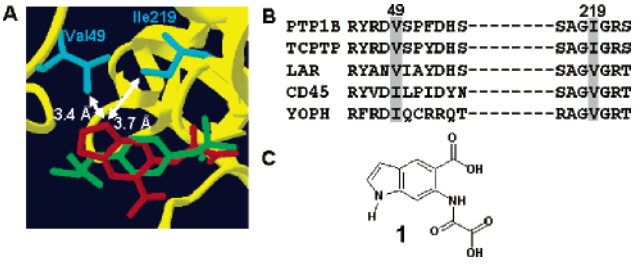

Figure 1. Engineering a PTP/inhibitor interface. (A) Crystal structure of PTP1B bound to compound **1** (red).[3] To highlight the binding mode of **1**, the PTP1B/**1** co-crystal structure was overlaid with a PTP1B/phosphopeptide co-crystal structure;[4] phosphotyrosine is shown in green. (B) Sequence alignment of some classical PTPs.[2] (C) Structure of compound **1**.

Table 1. Kinetic Constants for Inhibitor-Sensitized PTP1B Mutants

*p*NPP, pH 5.2	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
wild-type PTP1B	12 ± 1.8	0.32 ± 0.067	40
V49G PTP1B	1.1 ± 0.071	3.3 ± 0.14	0.33
I219G PTP1B	0.66 ± 0.014	2.8 ± 0.28	0.24
V49A/I219A PTP1B	0.90 ± 0.28	4.1 ± 1.2	0.22
V49A PTP1B	11 ± 3.7	2.2 ± 0.057	5.5
I219A PTP1B	24 ± 1.3	1.4 ± 0.35	17
DAD, pH 7.0	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})
wild-type PTP1B	16 ± 1.7	8.6 ± 1.8	1.86
V49A PTP1B	9.3 ± 2.8	97 ± 25	0.096
I219A PTP1B	26 ± 3.5	133 ± 6.1	0.194

PTP/derivatized inhibitor pair. In accordance with this strategy, N-substituted analogues of **1** (compounds **5a**−**k**) were prepared (Scheme 1).[6]

Compound series **5** was screened for mutant-selective PTP1B inhibition at 25 μM (Figure 2). Nine of the eleven compounds from the panel (**5a**−**i**) exhibited varying degrees of selectivity for at least one of the alanine mutants. As a rule, selectivity was greater for I219A PTP1B than that for V49A. The most selective mutant/inhibitor pairs consisted of I219A PTP1B and the *N*-methyl analogue of **1** (**5a**) and, to a lesser extent, the *N*-ethyl analogue (**5b**). To more accurately determine the level of selectivity for I219A PTP1B, the inhibitory constants (K_Is) were determined for the most selective enzyme/inhibitor pair identified in the initial screen. The K_I value of compound **5a**, assayed at pH 5.2, with wild-type PTP1B is 11 μM, and with I219A PTP1B, it is 1.1 μM, a 10-fold selectivity for I219A PTP1B over wild-type PTP1B. It has been shown previously that compound **1** more potently inhibits PTP activity at acidic pH than at physiological pH.[3] This is true for the analogues in panel **5**, as well. However, the I219A target specificity of compound **5a** is retained at pH 7.4: K_I (wild-type PTP) > 1000 μM; K_I (I219A PTP1B) = 43 μM.

A key advantage of using protein/inhibitor engineering for targeting PTPs is that due to the conserved nature of PTP domains, the approach should be applicable across the family of classical PTPs. The initial expansion of the sensitization process was applied

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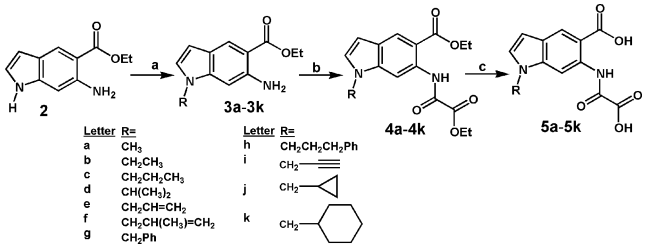
Scheme 1. Synthesis of Allele-Specific PTP Inhibitors[a]



[a] Compound **2** was synthesized essentially as described.[6] Conditions: (a) NaH (1.3 equiv), DMF, RX; (b) ethyl oxalyl chloride, THF; (c) i. NaOH, H_2O, EtOH; ii. HCl.

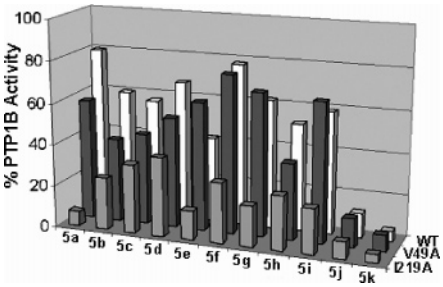


Figure 2. Screen of compound panel **5** for selective inhibition of engineered PTP1B mutants. The indicated compounds (25 μM) were incubated at 22 °C with 50 mM NaCl, 100 mM NaOAc (pH 5.2), *p*NPP (concentration corresponding to the K_M for the particular enzyme), and wild-type (background), V49A (middle), or I219A (foreground) PTP1B (as GST fusion proteins). Percent PTP1B activities in the presence of the inhibitors (normalized to a no-inhibitor control) are shown as bars.

to T-cell PTP (TCPTP), PTP1B's closest homolog.[2] Like PTP1B, TCPTP has a valine residue at position 49 and an isoleucine residue at position 219 (Val51 and Ile220, respectively, in TCPTP numbering; Figure 1B). Therefore, we predicted that inhibitors that are selective for inhibitor-sensitive PTP1B mutants, such as **5a**, would also be selective for the correspondingly mutated TCPTP enzymes.

Kinetic evaluation of purified I220A TCPTP confirms that, as with PTP1B, a single alanine mutation at position 219 is well tolerated in the TCPTP active site. The I220A TCPTP catalytic constant ($k_{cat} = 14.5 \pm 6.7\ s^{-1}$) is within error of the wild-type value ($12.5 \pm 1.7\ s^{-1}$), and the Michaelis constant for DAD is increased only modestly (wild type $K_M = 34 \pm 5.4\ \mu$M; I220A $K_M = 124 \pm 23\ \mu$M). These data suggest that the 219 position of PTPs may be widely "engineerable", that is, alanine mutations at this position will not disrupt the activity of classical PTPs. We measured the K_I values of compound **5a** against WT TCPTP and I220A TCPTP. Indeed, **5a** is strongly selective for I220A TCPTP over the wild-type enzyme; its K_I values for wild-type and I220A TCPTP are 7.0 and 0.23 μM, respectively, representing 30-fold selectivity for the inhibitor-sensitized TCPTP (787 and 35 μM, respectively, at pH 7.4). Moreover, compound **5a** is 48-fold selective for I220A TCPTP over wild-type PTP1B. To our knowledge, no compounds described in the literature demonstrate substantial selectivity for TCPTP over PTP1B.

Engineering of the TCPTP/compound **1** interface led not only to greater specificity but also to increased inhibitor potency. The parent inhibitor (**1**) inhibits wild-type TCPTP at 3.1 μM. The I220A TCPTP/**5a** pair is, therefore, roughly 10-fold more potent than the wild-type TCPTP/**1** pair, presumably due to efficient packing between the *N*-methyl group of **5a** and the methyl group of the Ala220 side chain.

To test the breadth of the engineered selectivity, we further screened compound **5a** against the commercially available PTPs human LAR and *Yersinia pestis* YopH (Figure 1B). Compound **5a** also demonstrated substantial selectivity for the engineered mutants with respect to these PTPs, both of which possess valine at position 219. Wild-type LAR and YopH were inhibited less potently than wild-type TCPTP: K_I (LAR) = 21 μM, K_I (YopH) = 31 μM (pH 5.2). Compound **5a**, therefore, is at least 30-fold selective for I220A TCPTP over every wild-type PTP tested to date.[7]

It is striking that compound **5a** is more potent and selective for I220A TCPTP than for I219A PTP1B. No modeling on TCPTP was necessary; the enzyme/inhibitor interface designed on PTP1B was simply transferred to TCPTP using primary sequence alignments, affording PTP selectivities that have not been attainable with conventional inhibitor screening methods.[8] Due to the conservation of the PTP active site, the PTP sensitizing strategy described here can likely be applied to many other PTPs, with no need to redesign the inhibitor interface for each new enzyme target. A widely applicable inhibitor sensitization approach will allow for the systematic targeting of PTPs in cellular studies and for the deconvolution of protein phosphorylation pathways.

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Supporting Information Available: Complete experimental and synthetic protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Compound **5a** also demonstrated a 50% inhibitory concentration of 35 μM against human CD45, at 0.3 mM *p*NPP, pH 5.2; a K_I value for this enzyme was not obtainable due to low activity of the commercial enzyme.

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