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Engineering non-natural inhibitor sensitivity in protein tyrosine phosphatase H1

Elizabeth R. Blair, Hillary E. Hoffman and Anthony C. Bishop*

Department of Chemistry, Amherst College, Amherst, MA 01002, USA

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Abstract—Protein tyrosine phosphatase H1, a member of the ubiquitous protein tyrosine phosphatase (PTP) superfamily of enzymes, is an important signaling molecule, mutant forms of which have been found in human colorectal cancers. Selective PTPH1 inhibitors would be valuable tools for investigating PTPH1's roles in cellular regulation. However, no PTPH1-specific inhibitors are known. To identify target-selective inhibitors of human PTPH1, we have redesigned a PTPH1/inhibitor interface. Structure-based protein design was used to identify two amino-acid residues, isoleucine 846 and methionine 883, that control PTPH1's sensitivity to oxalylaminoindole PTP inhibitors. Mutation of residues 846 and 883 to alanine and glycine, respectively, conferred novel inhibitor sensitivity onto PTPH1. From a small panel of putative inhibitors, compounds that potently and selectively target the inhibitor-sensitized PTPH1 mutants were identified.
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1. Introduction

The eukaryotic protein tyrosine phosphatase (PTP) superfamily comprises a large and diverse set of signaling enzymes, roughly 100 of which are encoded in the human genome.[1] The PTPs catalyze the dephosphorylation of phosphotyrosine residues in protein substrates and thus regulate levels of cellular tyrosine phosphorylation. Genetic and biochemical experiments have shown that PTP function is highly specific and tightly controlled.[2] Moreover, PTP activity can exert either positive or negative effects on a signaling pathway.[3,4] A complete understanding of eukaryotic signal transduction will thus require a full accounting of PTP function, and small molecules that specifically inhibit individual PTPs would facilitate the achievement of this far-reaching goal.

The search for PTP inhibitors with high target-specificity has intensified in recent years.[5] However, the overwhelming majority of PTP inhibitor development has focused on a single enzyme: the type-II-diabetes drug target, PTP1B.[6] A paucity of literature exists concerning inhibitors of other PTPs, even though many of these enzymes have critical signaling functions, and several have been implicated as potential therapeutic targets.[1]

Protein/small-molecule interface engineering has been used in a variety of biochemical systems to generate highly specific bioactive molecules.[7,8] Examples of such strategies include the generation of allele-specific protein-kinase substrates[9] and inhibitors;[10] the elucidation of myosin-1c's role in hair-cell adaptation;[11] the design of cell-specific inhibitors of calcineurin[12] and protein methyltransferases;[13] the generation of mutant-specific FKBP-binding chemical dimerizers;[14] and the development of hormone receptors with novel ligand specificities and activation mechanisms.[15–17] These seemingly disparate studies share a common feature: the mutagenic introduction of chemical diversity into a target protein receptor, to yield specific ligand/receptor pairs.

Large gene families that encode homologous enzymes present a particularly challenging problem for the discovery of target-specific inhibitors, and thus represent particularly attractive targets for protein/small-molecule engineering strategies.[10,13] The crux of such approaches lies in the introduction of functionally silent point mutations, which serve to create a novel binding pocket in the target enzyme. These mutations render the 'degeneracy' problem much more tractable; the active site of the mutated enzyme is sufficiently different from those of

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* Corresponding author. Tel.: +1 413 542 8316; fax: +1 413 542 2735; e-mail: acbishop@amherst.edu

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its off-target competitors to target with relatively few putative inhibitors.[10]

We have recently described a protein/small-molecule engineering approach for the design of selective PTP inhibitors.[18] Site-directed mutagenesis of a prototypical phosphatase, PTP1B, was used to generate inhibitor-sensitized PTP1B variants. From a small panel of inhibitors, compounds that selectively inhibit mutated forms of PTP1B over the wild-type enzyme were identified. The corresponding mutations also conferred novel inhibitor sensitivity to PTP1B's closest homolog, T-cell PTP (69% identity in the PTP catalytic domain).[18] These findings suggest that multiple PTPs may be amenable to sensitization through analogous mutagenic strategies.

In the current work, we have set out to engineer novel inhibitor sensitivity in PTPH1, a biologically important PTP that is less closely related to the PTP1B prototype than TCPTP (36% catalytic domain identity). While the full range of PTPH1 substrates has not been elucidated, it is known that PTPH1 is a crucial and wide-ranging signaling molecule. PTPH1 is the major PTP capable of dephosphorylating the immune tyrosine-based activation motifs present in the T-cell receptor ζ-subunit;[19] PTPH1 activity negatively regulates the tumor necrosis factor α-convertase;[20] and PTPH1 dephosphorylates p97, stabilizing its association with membranes in transitional endoplasmic reticulum assembly.[21] Moreover, a recent report has brought the connection between PTPH1 and human cancer to the fore. In a sequence analysis of all human PTPs in colorectal cancers, several somatic mutations in PTPH1 were discovered, suggesting that PTPH1 may act as a tumor suppressor.[22]

PTPH1 has not been subjected to targeted inhibitor-discovery studies, and no PTPH1-selective inhibitors are known. Here we present the first application of enzyme/inhibitor engineering to PTPH1, with the goal of identifying small molecules that are capable of selectively inhibiting the activity of sensitized PTPH1 variants, with respect to wild-type PTP activities (Fig. 1). Successful identification of such compounds would provide a means for chemically probing the roles of PTPH1 in cellular homeostasis, regulation, and/or oncogenesis.

2. Results and discussion

2.1. Design of allele-specific PTPH1 inhibitors

To design small-molecule inhibitors that are selective for rationally sensitized PTPH1 variants, we used the following criteria: the parent inhibitor must inhibit wild-type PTPH1, and the binding orientation of the inhibitor in the PTPH1 active site must be readily predictable. Although the three-dimensional structure of PTPH1 has not been reported, we hypothesized that active-site-directed PTP inhibitors would bind to PTPH1 in orientations analogous to their respective PTP1B-binding modes, owing to the conserved active-site structure in 'classical' PTPs. Under this assumption, we investigated a series of derivatized inhibitors based on the known PTP inhibitor 6-(oxalylamino)-1*H*-indole-5-carboxylic acid (compound **1**, Fig. 2). Oxalylaminoindole carboxylic acids inhibit a variety of classical PTPs, including PTPH1.[23,24] Importantly, the crystal structure of **1** bound to PTP1B has been reported, allowing for the possibility of structure-based design that exploits compound **1** as a scaffold from which to append synthetic modifications.[18,23] Specifically, we hypothesized that the indole nitrogen of **1** (*N*-1) could be modified without disrupting the inherent activity of the oxalylaminoindole platform (because it does not participate in hydrogen-bonding networks). Based on this analysis, we prepared a panel of *N*-1-substituted analogs of **1** (compound series **2a**–**2j**, Fig. 2). The synthesis of this 10 compound library has been reported previously.[18]

2.2. Sensitization of PTPH1 through mutations at isoleucine 846 and methionine 883

To generate non-natural inhibitor selectivity in the PTPH1 active site, it is necessary to introduce a small amino acid in a position that is occupied by a larger amino acid in the wild-type enzyme. A rational approach to such an undertaking is complicated by the fact that the PTPH1 catalytic-domain structure is not known. However, all reported structures of classical PTP catalytic domains share a common fold, and primary sequence alignments between the PTPH1 catalytic domain and the catalytic domain of the well-characterized PTP1B can be readily generated, allowing us to use PTP1B as a proxy for structure-based design on PTPH1 (Fig. 3).

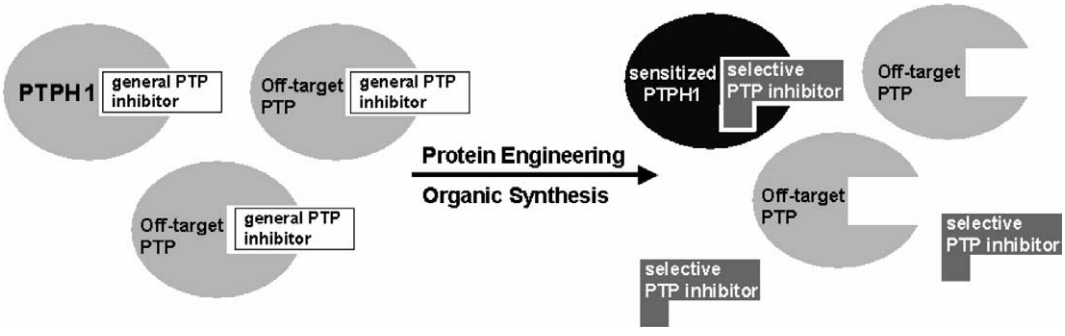

Figure 1. Schematic diagram of protein/small-molecule engineering approach for the inhibitor sensitization of PTPH1.

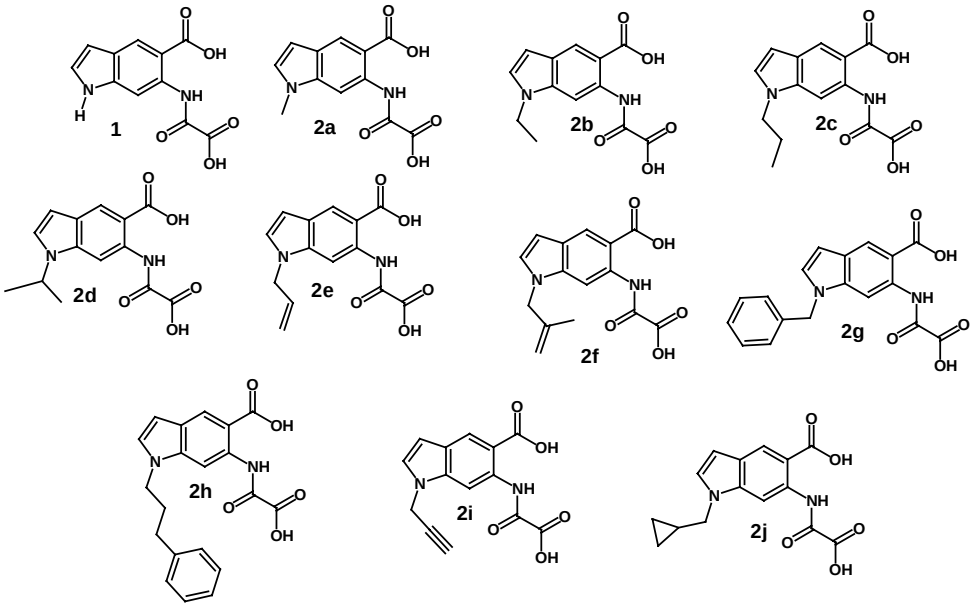

Figure 2. Chemical structures of compound **1** and compound panel **2a**–**2j**.

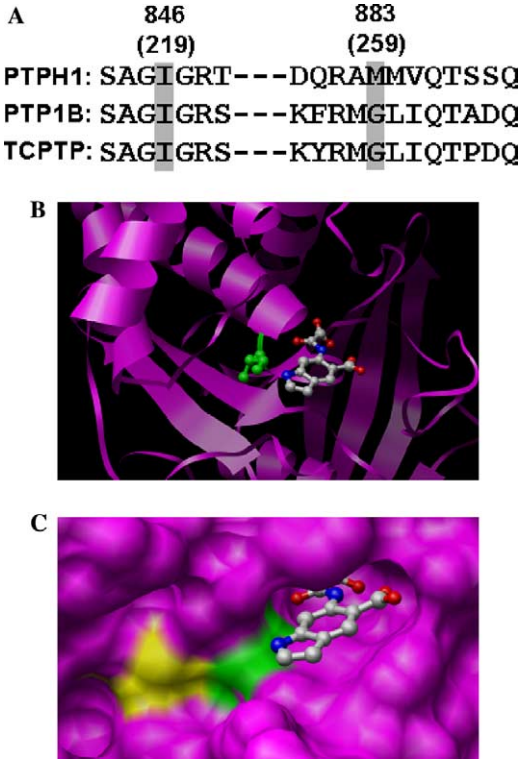

Figure 3. Engineering the PTPH1 active site using PTP1B as a prototype. (A) Partial sequence alignment of PTPH1, PTP1B, and TCPTP. Numbering is based on human PTPH1 with human-PTP1B numbering provided parenthetically. (B) Crystal structure of PTP1B (magenta) bound to compound **1**.[23] Compound **1** is colored by atom type: gray for carbon, red for oxygen, and blue for nitrogen. The side chain of isoleucine 219, which corresponds to isoleucine 846 of PTPH1, is shown in green. (C) Surface representation of PTP1B bound to compound **1**.[23] The PTP1B protein surface is shown in magenta, with the portions of the surface comprising isoleucine 219 (green) and glycine 259 (yellow, corresponds to methionine 883 of PTPH1) highlighted. Compound **1** is colored as in (B). The images in (B) and (C) were generated using the Chimera software package (http://www.cgl.ucsf.edu/chimera).

Guided by PTP1B structures, we selected isoleucine 846 (human PTPH1 numbering) as a candidate site for a first generation of sensitized PTPH1 mutants. The isoleucine 846 residue corresponds in sequence alignments to isoleucine 219 of PTP1B, a residue that we have previously shown to govern PTP1B's inhibitor sensitivity (Fig. 3A).[18] The δ-methyl group of PTP1B's isoleucine 219 is positioned 3.7 Å from the indole nitrogen of **1**, suggesting that a space-creating alanine mutation at the corresponding position in PTPH1 (I846A) may render PTPH1 susceptible to inhibition by members of compound series **2** (Fig. 3B). (We have avoided an isoleucine to glycine mutation, as a glycine mutation at the analogous position in PTP1B is deleterious to PTP activity.[18]) Additionally, large hydrophobic residues are present at position 846 in many other PTPs (either valine or isoleucine in 28 of 37 human classical PTP domains[25]), increasing the likelihood that an I846A PTPH1 mutant will possess inhibitor-sensitivity that is rare, or unique, among PTP enzymes. (Other residues that occupy position 846 position include cysteine in BDP1, LyPTP, and PEST; threonine in PTPλ; and alanine in PTPIA2, PTPIA2β, PTPκ, PTPμ, and PTPρ. None of these alanine-containing PTPs have been subject to oxalylaminoindole-based inhibitor studies. Thus, it is difficult to predict the sensitivity of these enzymes to members of compound series **2**.)

We also targeted a second PTPH1 active-site residue, methionine 883, whose mutation could confer additional inhibitor sensitivity onto PTPH1. In the PTP1B crystal structure, it has been observed that the surface of position 883 (259 in human PTP1B numbering) forms a contiguous surface with the 846 position, suggesting that an optimal 'sculpting' of the PTPH1 active site might require modification of either, or both, sites (Fig. 3C). The size and structure of amino-acid side chains found at position 883 vary widely among classical PTPs. For example, PTP1B possesses a glycine residue at position

883, whereas this position is occupied by amino acids as large as tyrosine in other PTPs.[25] (It has been shown previously that this 'natural hole' in PTP1B can be exploited in the design of selective PTP1B inhibitors.[26,27]) We hypothesized that mutation of PTPH1's methionine 883 to alanine or glycine may optimize the novel inhibitor sensitivity of engineered PTPH1 by creating a larger pocket to accommodate the 'bumps' present in compound series **2**. Such mutations may be particularly useful when combined with a mutation at position 846, as only two human PTPs, PTPIA2 and PTPIA2β, naturally possess the 'small/small' combination of alanine at position 846 and alanine or glycine at position 883 (glycine in both cases). To analyze the inhibitor-sensitization effects of positions 846 and 883 independently and in combination, we generated the following PTPH1 mutants: I846A; methionine 883 to alanine (M883A); methionine 883 to glycine (M883G); and two double mutants, I846A/M883A and I846A/M883G.

2.3. Characterization of inhibitor-sensitized PTPH1 mutants

The determinants for PTP cellular substrate-specificity generally lie outside of the active site,[25] making it unlikely that the I846- or M883-mutant enzymes would possess significantly altered cellular substrate specificity. However, these mutations could potentially disrupt the catalytic activity of PTPH1. Therefore, all of the PTPH1 mutants were thoroughly evaluated for in vitro catalytic activity. We purified the wild-type and mutant PTPH1 catalytic domains from *Escherichia coli* as His_6-fusion proteins and determined their catalytic properties (k_{cat} and K_M) using the artificial substrate *para*-nitrophenylphosphate (*p*NPP).[28,29]

We measured K_M (*p*NPP) and k_{cat} values for wild-type PTPH1 of 0.465 mM and 4.15 s^{-1}, respectively, in good agreement with previously reported values.[24] As shown in Table 1, the catalytic competencies of the mutant PTPH1 enzymes are modified only modestly. All of the PTPH1 mutants turn over substrate at rates that are comparable to that of wild-type PTPH1, while the K_M values for *p*NPP of the mutants that contain the I846A mutation are approximately 6- to 8-fold higher than the wild-type value. The catalytic efficiencies (k_{cat}/K_M) of all of the mutants are within a factor of 4 of the wild-type value, leading us to surmise that these enzymes are suitable for an allele-specific inhibitor-design strategy that requires a silent mutation.

Table 1. Kinetic constants for PTPH1 mutants with *para*-nitrophenylphosphate as substrate

Enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
PTPH1	4.15 ± 0.15	0.465 ± 0.040	8.92
I846A PTPH1	11.03 ± 0.97	2.79 ± 0.58	3.95
M883A PTPH1	4.31 ± 0.51	0.821 ± 0.28	5.25
M883G PTPH1	2.31 ± 0.05	0.346 ± 0.054	6.69
I846A/M883A PTPH1	8.40 ± 1.66	3.83 ± 1.10	2.19
I846A/M883G PTPH1	10.95 ± 0.87	2.72 ± 0.63	4.03

Table 2. Kinetic constants for inhibitor-sensitized PTPH1 mutants with the phosphopeptide DADEpYLIPQQG as substrate

Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})
PTPH1	5.92 ± 1.32	4.91 ± 2.99	1.21
I846A PTPH1	16.9 ± 1.9	21.7 ± 3.9	0.78
I846A/M883G PTPH1	22.1 ± 3.8	13.8 ± 2.1	1.61

We further characterized the PTPH1 enzymes that demonstrated the highest degrees of novel inhibitor sensitivity (I846A and I846A/M883G PTPH1, see below) by measuring their dephosphorylation activities with a phosphopeptide substrate (DADEpYLIPQQG). A truncated form of this peptide, which is derived from an autophosphorylation site (Tyr^{992}) of the epidermal growth factor receptor (EGFR), has been previously shown to be an efficient substrate for wild-type PTPH1.[24] As shown in Table 2, the catalytic efficiencies of I846A and I846A/M883G PTPH1 with DADEpYLIPQQG are modified only very slightly, with respect to wild-type PTPH1 (wild-type PTPH1: $k_{cat}/K_M = 1.21\ s^{-1}\ \mu M^{-1}$; I846A PTPH1: $k_{cat}/K_M = 0.78\ s^{-1}\ \mu M^{-1}$; I846A/M883G PTPH1: $k_{cat}/K_M = 1.61\ s^{-1}\ \mu M^{-1}$). While it is impossible to exclude the possibility that these PTPH1 mutants possess altered substrate specificity, or to predict a priori what level of change in enzymatic efficiency is tolerable for a mutant to complement the function of a wild-type enzyme, it is unlikely that these modest effects of the I846A and I846A/M883G mutations would be reflected in a significant change in PTPH1's biological substrate recognition.

2.4. Inhibitor screening

In an effort to identify compounds that display selectivity for a mutant PTPH1 over the wild-type, the members of compound series **2** were screened for PTP inhibition at a concentration of 75 μM. Figure 4 shows the results of these experiments for wild-type and I846A PTPH1 (compare magenta and green bars). Eight of ten series-**2** compounds exhibited some degree of selectivity for the alanine mutant with respect to the wild-type enzyme, with compounds **2d** and **2j** representing the exceptions. For most of the compounds, this I846A-selectivity was modest. However, the fact that I846A PTPH1 generally demonstrates a heightened ability to bind *N*-substituted analogs of compound **1** validates the 'blind' design strategy through which I846 was mutated in the absence of a PTPH1 crystal structure. The observation that the *N*-methyl analog of **1** is the compound most selective for I846A PTPH1 lends further support to our use of the PTP1B structure as a prototype (**2a**, 61% wild-type PTPH1 activity compared to 21% I846A activity, Fig. 4). This same compound has been identified as a target-selective inhibitor of I219A PTP1B and I220A TCPTP.[18] These further data with the corresponding mutant of PTPH1 suggest that the Ala846/methyl interface may be a general motif that can be exploited to target many different PTPs, even ones that are not particularly homologous to the PTP1B prototype.

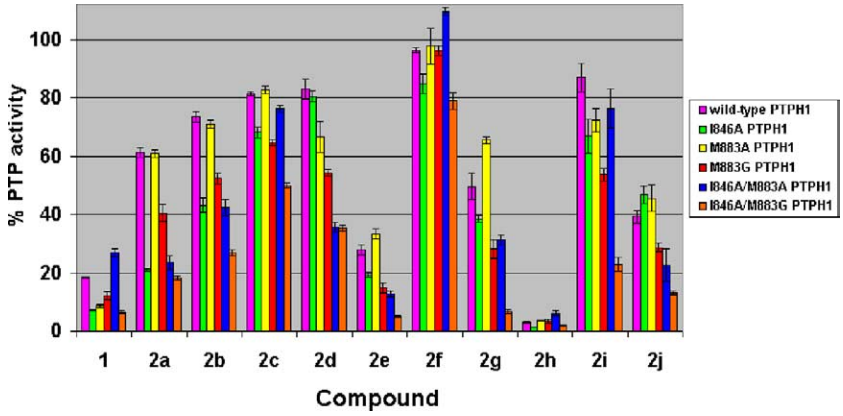

Figure 4. Screen of compound panel **2** for selective inhibition of PTPH1 mutants. The indicated compounds (75 μM) were incubated with wild-type PTPH1 (magenta), I846A PTPH1 (green), M883A PTPH1 (yellow), M883G PTPH1 (red), I846A/M883A PTPH1 (blue), or I846A/M883G PTPH1 (orange) and *p*NPP (concentration corresponding to the K_M for the particular enzyme, see Table 1). Percent PTPH1 activities in the presence of the inhibitors, normalized to no-inhibitor controls, are shown. Colored bars and error bars represent the average percent activities and standard deviations, respectively, derived from three experiments.

To more precisely determine the level of compound **2a**'s selectivity for I846A PTPH1, we measured its inhibition constant (K_I) for wild-type and I846A PTPH1. Compound **2a**'s selectivity for I846A PTPH1 over the wild-type enzyme is modest: The K_I value of compound **2a**, assayed at pH 7.0, with wild-type PTPH1 is 64 ± 7.0 μM, and with I846A PTPH1 it is 15 ± 1.5 μM—4-fold selectivity for the sensitized mutant. However, **2a** inhibits other wild-type PTPs much less effectively; its K_I value against wild-type TCPTP under these conditions is 388 ± 127 μM. Among a panel of PTPs assayed in previous work, TCPTP was the most susceptible to inhibition by **2a**.[18] Thus, in a mammalian cell line (or cell lysate, if members of compound series **2** prove to have low cellular activity or permeability), in which the gene encoding wild-type PTPH1 is replaced by I846A PTPH1, compound **2a** could potentially inhibit the sensitized PTPH1 gene product with at least 60-fold selectivity over every PTP tested to date.

To test the ability of position 883 to control PTPH1's inhibitor selectivity, we re-screened compound series **2** against M883A and M883G PTPH1 (Fig. 4, compare yellow and red bars, respectively, with magenta bars). The M883A mutation has very little effect, with regard to sensitization of PTPH1 to compound series **2**. By contrast, the M883G mutation serves as a broad, but relatively mild, sensitizer of PTPH1 to *N*-substituted analogs of **1**. Eight compounds from series **2** inhibited M883G PTPH1 more effectively than wild-type PTPH1 (compounds **2f** and **2h** showed no M883G-selectivity). Interestingly, some compounds from panel **2** demonstrate markedly different selectivity characteristics with the M883G mutant than observed for the I846A mutant. For example, the *N*-cyclopropylmethyl analog **2j** selectively inhibits M883G PTPH1, but demonstrated no selectivity for the I846A mutant. Moreover, the most M883G-selective inhibitor identified from compound panel **2** is the *N*-propargyl compound, **2i** (87% wild-type PTPH1 activity compared to 54% M883G activity in the presence of 75 μM **2i**), a compound that was not selected as a 'hit' from screens of other PTPs sensitized at the 219/846 position.[18]

We next asked whether the two modest sensitization effects of the I846A and M883G mutations could be combined to yield a PTPH1 whose inhibitor sensitivity differs more substantially from that of the wild-type. To identify any such synergistic effect of the two mutations, we screened compound series **2** against the double mutant, I846A/M883G PTPH1 (Fig. 4, orange bars). (For completeness, we also screened the I846A/M883A double mutant, but as predicted from the single-mutant data, its engineered inhibitor sensitivity was inferior to that of I846A/M883G PTPH1.) As shown in Figure 4, the combination of the I846A and M883G mutations considerably increases the engineered inhibitor sensitivity of PTPH1. All 10 members of compound series **2** demonstrate selectivity with respect to wild-type PTPH1 (Fig. 4, compare orange bars to magenta bars). Moreover, the potencies for I846A/M883G PTPH1 of five of the compounds (**2a**, **2e**, **2g**, **2h**, and **2j**) match or exceed the potency of the parent inhibitor (**1**) for wild-type PTPH1. To a first approximation, the inhibitor-sensitization effects of the I846A and M883G mutations are additive—that is, the two modest individual mutation effects give rise to more pronounced selectivities in the double mutant. An exception to this general trend is compound **2j**, which, despite having no special affinity for the I846A mutant, is more selective for the double mutant than the single M883G mutation. As observed with other 846/219-alanine mutants, the *N*-methyl derivative **2a** is substantially selective for I846A/M883G PTPH1. However, in contrast with the I846A single mutant, **2a** is not the most selective inhibitor of I846A/M883G PTPH1. The most selective inhibitor from series **2** is compound **2i** (87% wild-type PTPH1 activity compared to 23% I846A/M883G activity at 75 μM). As shown in Figure 5, compound **2i** is a classic dose-dependent inhibitor of I846A/M883G PTPH1, and the strong preference of **2i** for the double-mutant active site holds over a range of inhibitor concentrations.

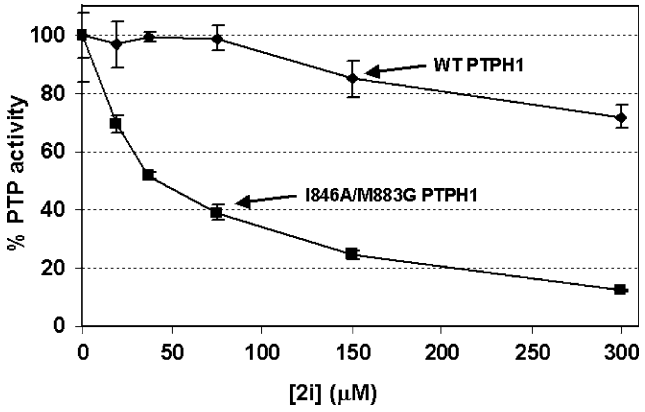

Figure 5. Selective, dose-dependent inhibition of I846A/M883G PTPH1 by compound **2i**. Various concentrations of **2i** were incubated with either wild-type (diamonds) or I846A/M883G PTPH1 (squares) PTPH1 and *p*NPP (concentration corresponding to the K_M for the particular enzyme, see Table 1). Percent PTPH1 activities, normalized to no-inhibitor controls, are shown. Error bars represent the standard deviations derived from three experiments.

It can be seen in Figure 4 that, despite compound **2i**'s high selectivity, it is not the most potent I846A/M883G PTPH1 inhibitor in compound series **2**; its high selectivity is due largely to its 'orthogonality' to the wild-type PTPH1 active site. Thus, we set out to more fully characterize the full range of potencies and selectivities that were present in the effective I846A/M883G PTPH1 inhibitors by measuring wild-type and I846A/M883G PTPH1 K_I values for the six most selective compounds from the inhibition screen (**2a**, **2e**, **2g**–**2j**, Table 3). In agreement with the 75 μM screen, compound **2i** is, indeed, the most selective inhibitor of I846A/M883G PTPH1 (26-fold selectivity with respect to wild-type PTPH1). The more potent **2a** is somewhat less selective (10-fold). Interestingly, several other compounds demonstrate selectivity that is only marginally lower than that of **2a**, with greater potency. Of particular interest is the *N*-3-phenylpropyl derivative **2h** (wild-type PTPH1: $K_I = 6.2$ μM; I846A/M883G PTPH1: $K_I = 0.87$ μM), a compound that inhibits wild-type PTPH1 more potently than the parent inhibitor (**1**, see Fig. 4), despite its bulky chemical appendage. At present, we do not understand the molecular basis for the heightened potency brought on by the 3-phenylpropyl group; it is possible that compound **2h** binds to PTPH1 in an orientation that is somewhat distinct from that of compound **1**. Regardless, compound **2h**, which is almost an order of magnitude more potent than compound **2a** for I846A/M883G PTPH1, and only slightly less selective, could serve as a lead for more directed libraries of future mutant-selective PTP inhibitors.

Table 3. Inhibition constants and selectivity factors for I846A/M883G-selective PTPH1 inhibitors

Compound	K_I (μM)		Selectivity K_I (WT)/K_I (I846A/M883G)
	PTPH1	I846A/M883G PTPH1	
2a	64 ± 7.0	6.2 ± 0.22	10
2e	17 ± 1.5	2.6 ± 0.14	6.5
2g	49 ± 9.4	5.7 ± 0.72	8.6
2h	6.2 ± 2.0	0.87 ± 0.16	7.1
2i	294 ± 34	11 ± 0.54	26
2j	41 ± 14	5.0 ± 0.54	8.1

The high hit rate of compound series **2** is notable, particularly considering that the inhibitor design for these compounds was performed on PTP1B—not PTPH1. No direct modeling on the PTPH1 catalytic domain was possible, due to the lack of a PTPH1 three-dimensional structure. Since alignments can be used to identify the corresponding mutations in any other classical PTP, these results suggest that the Ala846/Gly883 inhibitor-sensitization approach can be readily extended to other target phosphatases for which crystal structures have not been obtained. Moreover, the small number of compounds in panel **2** suggests that inhibitors which possess improved potency and selectivity will be achievable through synthetic optimization of the hits from panel **2**.

2.5. Evaluation of selectivity against other wild-type PTPs

To further evaluate the scope of the novel inhibitor sensitivity in I846A/M883G PTPH1, we screened the most potent and the most mutant-selective compounds, **2h** and **2i**, respectively, against wild-type PTP1B and TCPTP. These PTPs naturally possess glycine at position 883, but they each have isoleucine at position 846 (Fig. 3A). If, as the PTPH1 data suggest, the Ala846/Gly883 combination is required for full sensitivity to members of panel **2**, we would expect that wild-type PTP1B and TCPTP are not sensitive to inhibition by **2h** and **2i**. Indeed, as shown in Table 4, the sensitivities of wild-type PTP1B and TCPTP to **2h** and **2i** are very close to that of wild-type PTPH1. Although wild-type PTP1B is slightly more sensitive to **2h** and **2i** than PTPH1, substantial selectivity for I846A/M883G PTPH1 with respect to all three wild-type PTP activities is retained for both compounds. Compound **2h** is at least 6-fold selective for I846A/M883G PTPH1 over every wild-type PTP tested to date, and compound **2i** is at least 13-fold selective for I846A/M883G PTPH1 over every wild-type PTP tested to date. Notably, much larger selectivities were also observed. For example, **2i** inhibits I846A/M883G PTPH1 57-fold more potently than wild-type TCPTP.

Table 4. Wild-type PTP inhibition constants for compounds **2h** and **2i**

Enzyme	K_I (μM)	
	Compound **2h**	Compound **2I**
PTPH1	6.2 ± 2.0	294 ± 34
PTP1B	5.7 ± 0.85	154 ± 11
TCPTP	17 ± 3.4	645 ± 59

3. Conclusion

In an effort to identify selective inhibitors of PTPH1, a biologically important tyrosine phosphatase for which no selective inhibitors are known, we redesigned a PTPH1/small-molecule interface. Through structure-based engineering of the PTPH1 active site, we identified two residues, isoleucine 846 and methionine 883, which control the sensitivity of PTPH1 to active-site-directed inhibitors. Mutation of either, or both, of these positions to small amino acids yielded PTPH1 variants with increased inhibitor sensitivity. Screening of an oxalylaminoindole compound panel against the inhibitor-sensitized PTPH1 mutants was used to identify compounds that target the sensitized PTPH1 selectively. The sensitized PTPH1/inhibitor pairs described here provide the first chemical means for probing PTPH1 function in the presence of other PTP activities.

4. Experimental

4.1. Inhibitor synthesis

Compound **1** was prepared essentially as described.[23,30] Compounds **2a**–**2j** were prepared as described.[18]

4.2. Plasmid construction and site-directed mutagenesis

A plasmid for the expression of the PTPH1-His_6 catalytic domain (residues 623–913) was generated by cloning the PTPH1 catalytic domain into the pET-21b plasmid from the plasmid pFLAG-CMV2-PTPH1Δ4.1 (Carl P. Blobel, Memorial Sloan Kettering Cancer Center).[20] The primers used for cloning were (5′–3′) atcctgaattcgt gtccggagggtggggacac, which contains an *Eco*RI restriction site, and atcctaagcttgttgtcttaactaggatccagc, which contains a *Hin*dIII restriction site. PCR amplification of the PTPH1 catalytic domain was carried out on pFLAG-CMV2-PTPH1Δ4.1 using these primers and *Pfu* polymerase (Stratagene) according to the manufacturer's instructions. The PCR product and pET-21b were doubly digested with *Eco*RI and *Hin*dIII, gel purified, and ligated using T4 DNA ligase. The presence of the PTPH1 insert was confirmed by restriction digest with *Eco*RI and *Hin*dIII, and by sequencing (Cornell Biotechnology Resource Center). The PTPH1 stop codon upstream of pET-21b's His_6-encoding region was removed using the Quikchange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions, with the following primers (stop codon converted to a serine codon; mutagenic bases are capitalized): gctggatcctagttCagacaacCagcttgcggccg and cggccgcaagct GgttgtctGaactaggatccagc. Inhibitor-sensitizing mutations were introduced using the Quikchange kit and the following primers: hPTPH1-I846A1, gttcactg-cagtgctggCGCCggtcgaaccggtgtgttggtc; hPTPH1-I846 A2, gaccaacacaccggttcgaccGGCCgccagcactgcagtgaac; hPTPH1-M883A1, gaccagcgcgccGCgatggtgcagacGtcaa gccag; hPTPH1-M883A2, ctggcttgaCgtctgcaccatcGCg gcgcgctggtc; hPTPH1-M883G1, gcgagaccagcgcgccGG-Catggtgcagacatcaag; hPTPH1-M883G2, cttgatgtctgcac-catGCCggcgcgctggtctcgc. The desired mutations were confirmed by sequencing (Cornell Biotechnology Resource Center).

4.3. Expression and purification of PTPs

The PTPH1-His_6-encoding plasmids were transformed into BL21 (DE3) *E. coli*. Single colonies were picked and used to inoculate 500 mL LB cultures, which were grown to mid-log phase, and induced with 0.2 mM IPTG for 5 h. Cultures were pelleted and frozen at −80 °C. Cell lysis was achieved by incubation of the cell pellet in BPER (Pierce) for 15 min, and purifications of PTPH1-His_6 were carried out using Ni–NTA resin (Stratagene) according to the manufacturer's instructions. The enzymes were stored at −20 °C in 50 mM 3,3-dimethylglutarate, pH 7.0, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, and 30% glycerol. Expression levels ranged from 3.2 to 5.6 mg enzyme per liter of culture. Protein concentrations were measured by the Bradford assay, and enzyme purities were estimated by SDS–PAGE. All mutant and wild-type PTPH1-His_6 preparations were estimated to be $\geqslant$95% pure. Wild-type PTP1B and TCPTP were expressed and purified as described.[18]

4.4. Kinetic characterization of PTPH1 variants

PTPH1 kinetic assays with *p*NPP were carried out at 22 °C in a total reaction volume of 200 μL, containing 50 mM 3,3-dimethylglutarate, pH 7.0, 50 mM NaCl, 1 mM EDTA, *p*NPP (WT: 0.3–3 mM, I846A: 0.7–8 mM, M883A: 0.3–5 mM, M883G: 0.31–10 mM, I846A/M883A: 2–23 mM, I846A/M883G: 0.625–20 mM), and the appropriate PTPH1 enzyme (26–54 nM). Between 4 and 14 min after enzyme addition, the reactions were quenched with 40 μL of 5 N NaOH. The quenched reaction mixtures (180 μL) were loaded onto a 96-well plate, and the absorbances at 405 nm were read with a Molecular Devices Versamax plate-reader. The kinetic constants for each enzyme were determined by fitting the data to the Michaelis–Menten equation. Values given in Table 1 represent the averages and standard deviations of at least three independent experiments.

PTPH1 kinetic assays with the phosphopeptide DAD-EpYLIPQQG (Calbiochem) were carried out by measuring increasing absorbance at 282 nm, essentially as described.[18,31] Assays were performed at 22 °C in a total reaction volume of 200 μL and contained the following: 50 mM 3,3-dimethylglutarate, pH 7.0, 125 mM NaCl, 1 mM EDTA, 100 μM DADEpYLIPQQG, and the appropriate PTPH1 enzyme (35–64 nM). The data were fit to the integrated Michaelis–Menten equation as described.[18,31] Values given in Table 2 represent the averages and standard deviations of at least three independent experiments.

4.5. Inhibition assays

Inhibition screens were performed with *p*NPP as the substrate in reaction mixtures equivalent to those

described above, with the exception that 4 μL DMSO (vehicle) or 4 μL of 3.75 mM inhibitor (dissolved in DMSO) was added. The *p*NPP concentration was normalized to each enzyme's K_M value for *p*NPP. The percent activity was found by comparing the absorbances of reactions with and without inhibitor.

Inhibition constant (K_I) values were determined through inhibition assays identical to those described above, with the exception that *p*NPP and inhibitor concentrations were varied. The K_I values were determined by fitting inhibition data to the Michaelis–Menten equation for competitive inhibition. Values reported in Tables 3 and 4 represent the averages and standard errors of at least four independent measurements that fit to the Michaelis–Menten equation with a correlation coefficient of $\geqslant 0.9$.

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