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A gatekeeper residue for inhibitor sensitization of protein tyrosine phosphatases

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Abstract—Allele-specific enzyme inhibitors are powerful tools in chemical biology. However, few general approaches for the discovery of such inhibitors have been described. Herein is reported a method for the sensitization of protein tyrosine phosphatases (PTPs) to small-molecule inhibition. It is shown that mutation of an active-site isoleucine to alanine (I219A) sensitizes PTP1B to inhibition by a class of thiophene-based inhibitors. This sensitization strategy succeeds for both 'orthogonal' inhibitors, designed to be incompatible with wild-type PTP active sites, and previously optimized wild-type PTP inhibitors. The finding that the I219A mutation sensitizes phosphatase domains to a variety of compounds suggests that isoleucine 219 may act as a 'gatekeeper' residue that can be widely exploited for the chemical–genetic analysis of PTP function.

Large gene families that encode homologous proteins represent particularly challenging cases for chemical biology. This is, in large part, due to the 'degeneracy' problem; it is difficult to chemically differentiate between active (or allosteric) sites that bear a high degree of structural similarity with one another. One, now well established, method for circumventing the degeneracy problem is through the engineering of protein/small-molecule interfaces—that is, through modification of a protein receptor's binding site, in addition to the complementary modification of a potentially selective small-molecule ligand.[1,2]

One powerful application of protein/small-molecule engineering is the generation of allele-specific enzyme inhibitors. Several critical enzyme families in the eukaryotic proteome, including protein kinases,[3] protein methyltransferases,[4] and phosphoinositide 3-kinases,[5] have been engineered to possess novel inhibitor sensitivity. This engineered sensitivity, not present in related wild-type enzymes, allows for the identification of selective inhibitors from relatively small panels of putative inhibitors. The enzyme-sensitization approach has proven to be particularly effectual for the protein kinases, as members of the kinase family possess a 'gatekeeper' residue that can readily be identified through protein sequence alignments.[6] Targeted mutation of the gatekeeper residue (to alanine or glycine) has led to the creation of a general strategy, termed chemical–genetic analysis, for the study of protein kinase function. Recently, this method has been used to elucidate the in vivo function of an impressive array of yeast kinases (e.g., Cdc28,[6,7] Ime2,[7] Pho85,[8] Fus3,[6] and Apg1[9]), as well as mammalian kinases (e.g., CamKII,[10] v-erbB,[11] and GRK2.[12]).

We have recently described the first example of inhibitor-sensitization for another large and important class of signaling enzymes: the protein tyrosine phosphatases (PTPs).[13,14] The engineering of a prototype phosphatase, the type II diabetes drug target PTP1B, was guided by the following criteria: an amino acid chosen for mutagenesis must be large enough that substitution by a small amino acid creates a novel binding pocket; the mutant PTP must retain catalytic activity; and the residue identified for PTP1B-sensitization must be present in other PTPs, eliminating the need to redesign the PTP/inhibitor interface for each target. Valine 49 and isoleucine 219 (V49 and I219, human PTP1B numbering) both meet these criteria.[13]

In a previous report, we utilized the indolic nitrogen of an oxalylaminoindole PTP inhibitor (compound **1**, Fig. 1a) as a 'hook' to which we attached groups that were designed to be incompatible with wild-type PTP active sites.[13] While this approach did yield a 10-fold selec-

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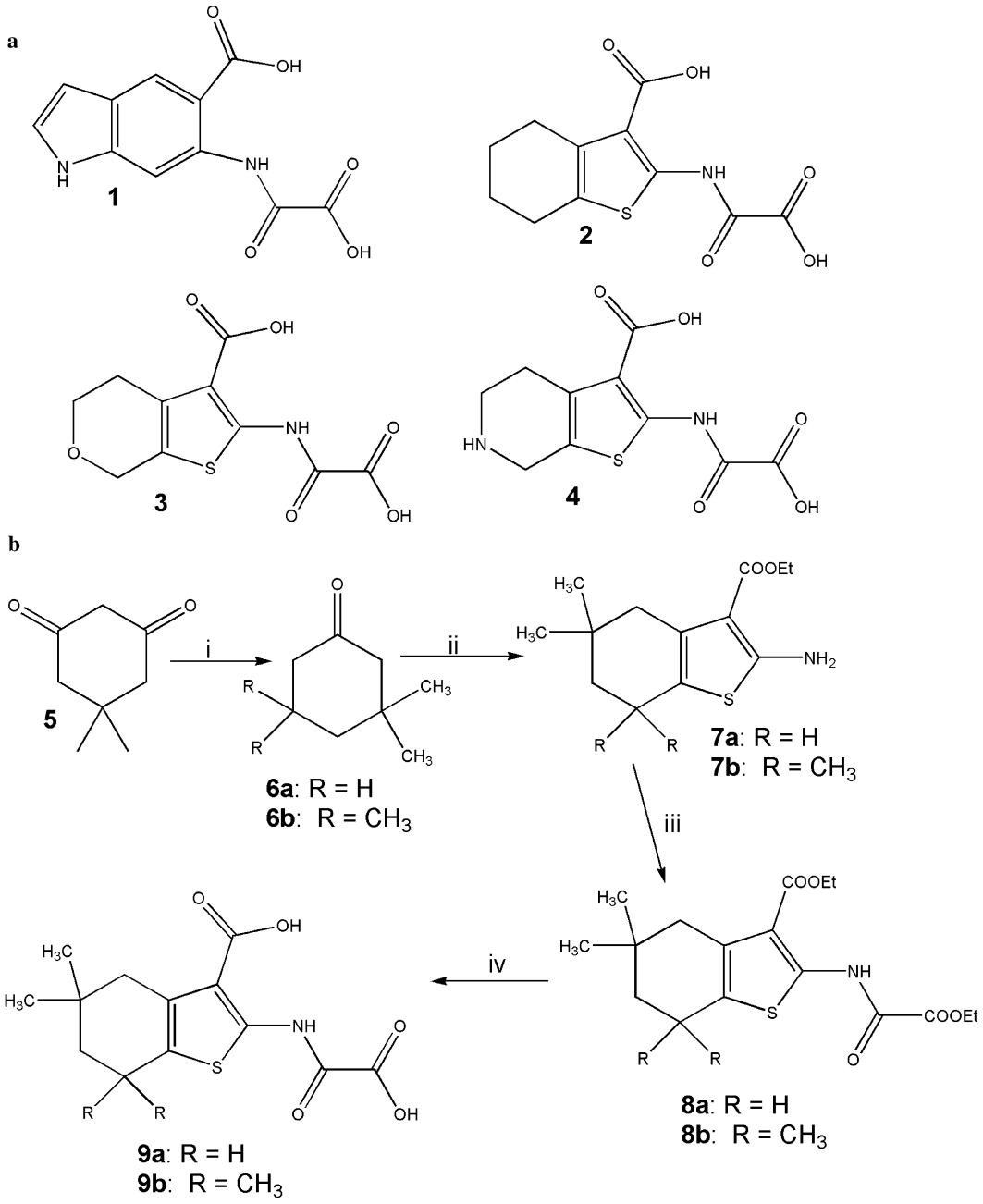

Figure 1. (a) Chemical structures of compounds **1**–**4**. (b) Chemical structures and synthetic schemes for compounds **9a** and **9b**. Reagents and conditions: (i) H_2, AcOH, Pd/C, cat. H_2SO_4; (ii) ethyl cyanoacetate, sulfur, morpholine, EtOH, reflux; (iii) ethyl oxalyl chloride, THF; (iv) NaOH, H_2O, EtOH.

tive inhibitor of a mutated PTP1B (I219A), it suffered from the inherent lack of potency of oxalylaminoindole PTP inhibitors at physiological pH.[13] Moreover, the indole-derivative inhibitors showed little to no selectivity for mutants of valine 49, a position that is particularly attractive for a general PTP inhibitor-sensitization strategy, as it is occupied by valine or isoleucine in 35 of the 37 classical PTP catalytic domains in humans. (Position 219 is occupied by either valine or isoleucine in 28 classical PTP domains.[15])

As a scaffold for a second generation of allele-specific PTP inhibitors, we selected 2-oxalylamino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (compound **2**, Fig. 1a).[16] Previous reports have shown that compound **2** effectively inhibits several PTPs, including PTP1B and its closest homolog, TCPTP, as well as phosphatases that are not especially homologous to PTP1B (e.g., PTPH1).[17–20] The three-dimensional structure of compound **2** bound to a PTP has not been reported; however, a crystal structure of a slightly less potent PTP inhibitor that differs from **2** only by the replacement of a methylene group with an oxygen atom (**3**, Fig. 1a) is known.[16] Surmising that compounds **2** and **3** bind to PTP active sites in identical orientations, we inspected the PTP1B/**3** structure for positions that could

be derivatized to target PTPs sensitized by mutations at valine 49 or isoleucine 219 (Fig. 2). Carbons 5 and 7 (C-5 and C-7) lie less than 5 Å away from the V49 and I219 side chains, respectively (Fig. 2). Based on this analysis, we hypothesized that analogs of **2**, derivatized at C-5 and/or C-7, may selectively target V49A and/or I219A PTP1B. In addition, extension of chemical groups 'up' and 'down' from the sp^3-hybridized carbons on the puckered six-membered ring of **2** appeared to hold promise for targeting the novel binding pocket of a V49A mutant, since V49 lies beneath the fused ring system. This analysis is consistent with previous findings that V49 mutants are not effectively targeted by flat indole-based inhibitors; in these inhibitors the chemical appendages extend 'sideways' from an sp^2-hybridized nitrogen atom, missing the engineered pocket of V49 mutants.[13]

We synthesized two 'bumped' inhibitors, compounds **9a** and **9b**, which were designed to exploit the novel binding pockets of the I219A and/or V49A mutants (Fig. 1b, see Supplementary data for synthetic details). These compounds contain dimethyl moieties at either C-5 (**9a**) or both the C-5 and C-7 positions (**9b**). (Due to synthetic difficulties, analogs with bumps exclusively at C-7 were not prepared.)

Using the artificial PTP substrate, *para*-nitrophenyl phosphate (*p*-NPP), compounds **9a** and **9b**, along with the parent molecule **2**, were assayed for selective inhibition of PTP1B activity at pH 7.0. Figure 3 shows the results of these inhibition screens for the GST-fusion proteins of wild-type PTP1B, V49A PTP1B, and I219A PTP1B. The C-5-derivatized analog, **9a**, shows an inhibitory profile that is essentially unchanged from the parent molecule (Fig. 3). Presumably the dimethyl moiety at C-5 simply extends out of the active site. Thus, **9a** was not investigated further. By contrast, the tetramethyl derivative, **9b**, shows the classical inhibitory profile for an 'orthogonal' design strategy: compared to the parent ligand, reduced potency with respect to the wild-type enzyme and comparable (V49A) or increased (I219A) potency with the sensitized enzymes (Fig. 3). To more accurately determine the level of selectivity for the sensitized PTP1B mutants, the inhibitory constant (K_I) values for **9b** with the wild-type and mutant enzymes were determined (Table 1). Compound **9b** is truly orthogonal with respect to wild-type PTP1B (K_I > 300 μM), and it demonstrates >10-fold selectivity for V49A PTP1B (K_I = 27 μM) and >30-fold selectivity for I219A PTP1B (K_I = 10 μM).

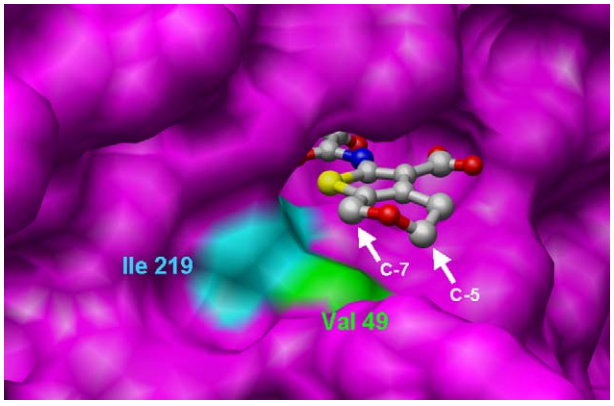

Figure 2. Surface representation of compound **3** bound to PTP1B (PDB: 1C87).[16] The PTP1B protein surface is shown in magenta, with the portions of the surface comprising Val49 (green), Ile219 (cyan) highlighted. Compound **3** is colored by element: gray for carbon, red for oxygen, blue for nitrogen, and yellow for sulfur. The arrows indicate the presumptive positions of C-5 and C-7 in the complex of compound **2** with PTP1B.

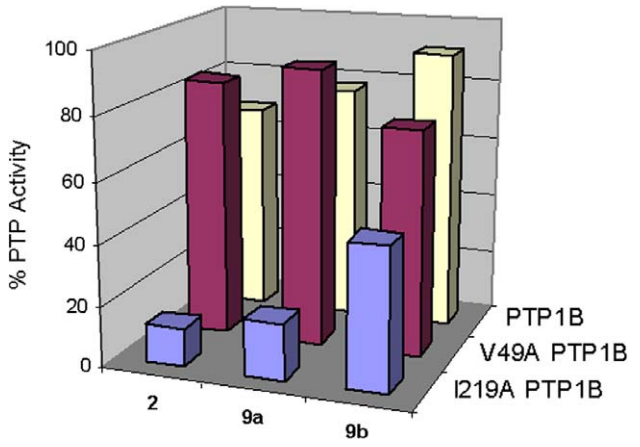

Figure 3. Selective inhibition of engineered PTP1B mutants by compounds **2**, **9a**, and **9b**. The indicated compounds (20 μM) were incubated with wild-type (background), V49A (middle), or I219A (foreground) PTP1B, and *para*-nitrophenyl phosphate (concentration corresponding to the K_M for the particular enzyme at pH 7.0: 2.08, 6.57, and 3.09 mM, respectively). Percent PTP1B activities in the presence of the inhibitors, normalized to a no-inhibitor (vehicle) control, are shown as bars. The bars represent the average value from three independent experiments, with relative standard deviations of less than 4%.

Compound **9b** is the first known compound to demonstrate substantial allele-specific character with a V49 PTP mutant. Since compounds **2** and **9a** show no allele-specific affinity for V49A PTP1B, it is clear that derivatization at C-7 is necessary for successful V49A targeting. These data may represent a useful starting place for future panels of more V49-directed inhibitors. However, the tetrahydrobenzothiophene scaffold will probably not be ideal for such an approach: V49A PTP1B is less potently inhibited by the parent molecule, **2**, than is wild-type PTP1B, suggesting that the V49 side chain makes important contacts with the six-membered ring of **2** that are lost upon mutation of V49 to alanine.

Surprisingly, strong I219A PTP1B-specific character was also observed for the parent molecule, **2** (Fig. 3

Table 1. Inhibition constants for compounds **2**, **9b**, and **4** on wild-type and sensitized PTP1B enzymes

Compound	Wild-type PTP1B K_I (μM)	V49A PTP1B K_I (μM)	I219A PTP1B K_I (μM)
2	34 ± 1.3	ND	1.0 ± 0.089
9b	>300	27 ± 1.8	10 ± 0.64
4	6.0 ± 0.34	ND	0.23 ± 0.0046

and Table 1). Compound **2** is, approximately, a 30-fold more potent inhibitor of I219A PTP1B than wild-type PTP1B (wild-type PTP1B: $K_I = 34\ \mu M$; I219 PTP1B: $K_I = 1.0\ \mu M$). Apparently, the I219 side chain of wild-type PTP1B precludes the optimal binding orientation of the fused thiophene-based inhibitors—regardless of whether or not the six-membered rings contain bumps that are added in an orthogonal approach. Moreover, it appears that the 219 position may act as a gatekeeper in PTPs: I219 controls access of inhibitors to the active site, whether or not the compounds are concertedly designed as 'allele-specific.'

To further test the idea that I219 may serve as a gatekeeper for many inhibitors, even potent wild-type inhibitors, compound **4** (Fig. 1a) was synthesized. It has been shown previously that the substitution of **2**'s C-6 methylene with nitrogen leads to increased potency for PTP1B and TCPTP.[16] A salt-bridge interaction between the introduced amino group and aspartate 48 (D48) increases the potency of **4** with respect to the closely related **2** and **3**, giving rise to substantial selectivity for D48-containing PTPs (e.g., PTP1B and TCPTP) over PTPs that contain other amino acids at this position (Fig. 4a).[16] We hypothesized that the potency and selectivity of this 'optimized' PTP inhibitor could be increased even further by sensitization of a target PTP. Indeed, compound **4** inhibits I219A PTP1B approximately 25 times more potently ($K_I = 0.23\ \mu M$) than it does wild-type PTP1B (Table 1). The **4**/I219A PTP1B K_I value of 230 nM is remarkably low for an active-site-directed PTP inhibitor with a molecular weight of less than 300 g/mol.[21] Moreover, at neutral pH, **4** is roughly 200-fold more potent than previously identified allele-specific PTP inhibitors.[13] Importantly, this heightened potency also gives rise to substantial selectivity not observed with wild-type PTPs. For example, **4** is only 2-fold selective for PTP1B with respect to PTP1B's closest homolog, TCPTP ($K_I = 10 \pm 0.74\ \mu M$)—a common and vexing problem among PTP1B inhibitors.[21] By contrast, **4** is 45-fold selective for I219A PTP1B over TCPTP, suggesting that **4** could be used in signaling studies with I219A PTP1B-expressing cells (or lysates) to specifically target PTP1B activity. Additionally, the gatekeeper functionality of position 219 is not limited to PTP1B. Introduction of the corresponding mutation in TCPTP switches the selectivity, rendering I220A TCPTP ($K_I = 0.71 \pm 0.030\ \mu M$) 8-fold more susceptible to inhibition by **4** than wild-type PTP1B ($K_I = 6.0\ \mu M$).

To test whether the enhanced potency of **4** could be used to selectively target sensitized PTPs in the context of complex proteomes, the activities of wild-type PTP1B- and I219A PTP1B-containing crude *Escherichia coli* lysates were assayed. Indeed, the selectivity of **4** in assays with purified enzyme translates directly to the lysate-catalyzed reaction. At 1 μM **4**, only slight inhibition of wild-type PTP1B is observed (Fig. 4b). By contrast, almost complete suppression of activity is observed in reactions containing I219A PTP1B lysate (Fig. 4c). Since wild-type PTP1B is (slightly) more susceptible to inhibition by **4** than is TCPTP, these experiments also suggest that the activity of a sensitized PTP1B could

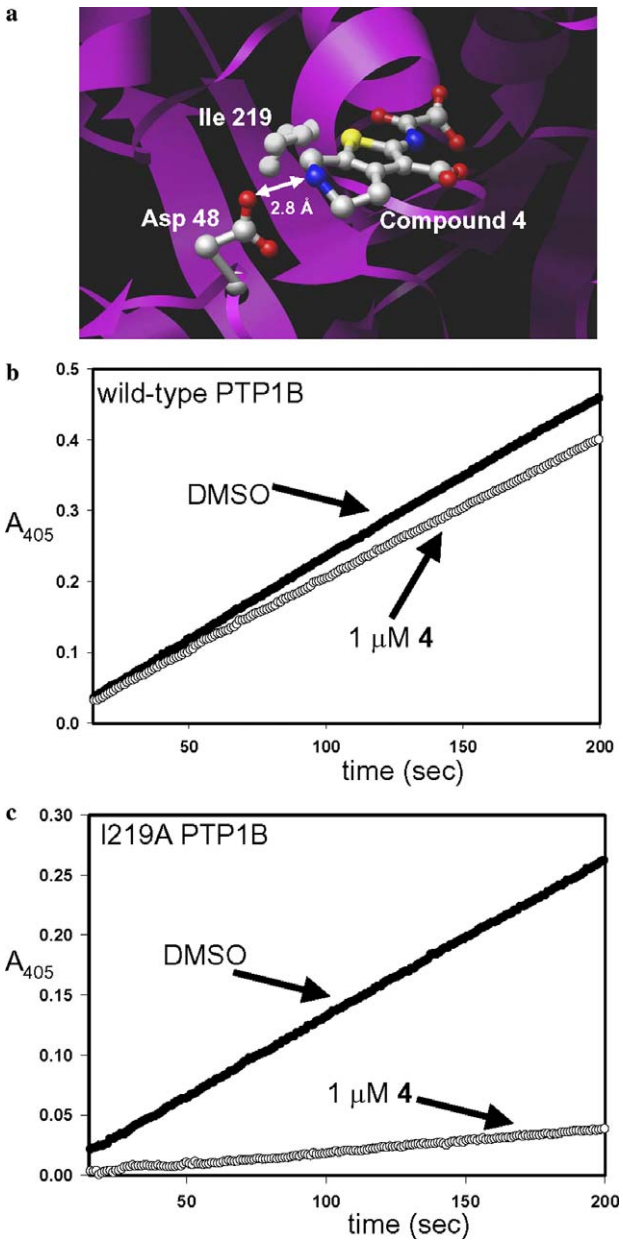

Figure 4. (a) Interaction of compound **4** with aspartate 48 in PTP1B (PDB: 1C88).[16] The PTP1B backbone is shown as a ribbon diagram in magenta. The Asp48 and Ile219 side chains and compound **4** are colored by element as in Figure 2. (b and c) Selective inhibition of I219A PTP1B in a complex proteome mixture. Crude cell lysate from wild-type PTP1B- (b) or I219A PTP1B-overexpressing (c) *Escherichia coli* was incubated with DMSO (vehicle, filled circles) or 1 μM **4** (open circles), and 0.5 mM *p*-NPP. Phosphatase activity was measured by monitoring the time-dependent increase in absorbance at 405 nm.

be ablated in a mammalian proteome without significantly disrupting TCPTP activity. Moreover, since **4** was previously optimized for TCPTP/PTP1B inhibition, the affinity of **4** for I219A PTP1B should be far greater than for any other PTP tested to date.[16]

The clear advantage of a successful allele-specific inhibitor approach, over medicinal chemistry, is in its generality: specificity for a given target can be straightforwardly engineered instead of 'hunted' for. However, the identifi-

cation of gatekeeper residues in an enzyme family is a key prerequisite for a widely applicable allele-specific approach. Our data show that I219 is an important gatekeeper for PTP inhibition. While the degree of sensitization varies from compound to compound, I219A mutant PTPs have proven to contain novel inhibitor sensitivity for many members of the two inhibitor families tested to date.[13,14] The current findings show that engineering of the gatekeeper residue can improve the potency and selectivity even of known compounds, heightening the utility of this approach, and possibly rendering synthesis of new compounds unnecessary for allele-specific PTP-inhibitor discovery. Our data, combined with recent findings of mutations that confer novel inhibitor resistance to PTPs,[22] will allow for the sculpting of PTPs with tunable inhibitor sensitivities. This combination of genetic manipulation and small-molecule control of PTP activity will facilitate the chemical–genetic analysis of PTP function in cells and model organisms.

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Supplementary data

Complete description of experimental protocols, synthetic procedures, and new-compound characterization. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.05.011.

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