2-(Oxalylamino)-Benzoic Acid Is a General, Competitive Inhibitor of Protein-tyrosine Phosphatases*

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Protein-tyrosine phosphatases (PTPs) are critically involved in regulation of signal transduction processes. Members of this class of enzymes are considered attractive therapeutic targets in several disease states, e.g. diabetes, cancer, and inflammation. However, most reported PTP inhibitors have been phosphorus-containing compounds, tight binding inhibitors, and/or inhibitors that covalently modify the enzymes. We therefore embarked on identifying a general, reversible, competitive PTP inhibitor that could be used as a common scaffold for lead optimization for specific PTPs. We here report the identification of 2-(oxalylamino)-benzoic acid (OBA) as a classical competitive inhibitor of several PTPs. X-ray crystallography of PTP1B complexed with OBA and related non-phosphate low molecular weight derivatives reveals that the binding mode of these molecules to a large extent mimics that of the natural substrate including hydrogen bonding to the PTP signature motif. In addition, binding of OBA to the active site of PTP1B creates a unique arrangement involving Asp181, Lys120, and Tyr46. PTP inhibitors are essential tools in elucidating the biological function of specific PTPs and they may eventually be developed into selective drug candidates. The unique enzyme kinetic features and the low molecular weight of OBA makes it an ideal starting point for further optimization.

Reversible tyrosine phosphorylation reactions play pivotal roles in most cellular signaling processes. Protein-tyrosine kinases phosphorylate cellular substrates on tyrosine residues and protein-tyrosine phosphatases (PTPs)1 remove phosphate from these residues (for reviews, see Refs. 1 and 2). It is generally believed that low molecular weight, selective PTP inhibitors may be useful in the treatment of a variety of diseases such as diabetes, autoimmunity, and cancer (1, 2).

Recent studies have provided important insight to some of the basic structural requirements for PTP-substrate and inhibitor interactions (reviewed in Refs. 3–8). It is particularly encouraging that highly selective, active site-directed PTP1B inhibitors have been reported (9). However, although significant progress has been made toward developing PTP inhibitors, most compounds have features that make them unsuitable as starting points for optimization to orally active drugs (for a recent review, see Ref. 6). As an example, peroxovanadium compounds have contributed significantly to our understanding of insulin signaling (10, 11), but appear to be too toxic and unspecific for use as drugs. Bi phosphonates, such as alendronate, have been shown to inhibit PTPs, but their inherent affinity for bone is likely to prevent their general use in other target tissues (12). Furthermore, several of these inhibitors are time-dependent and seem to act through covalent modification of the catalytic cysteine in PTPs (13, 14). The most specific inhibitors produced so far have either been phosphonates (9, 15) or those based on peptides that are not suited for clinical use due to their lack of oral bioavailability and metabolic instability (reviewed in Ref. 6).

The phosphate requirements of most of the previously described PTP inhibitors have their natural justification, in that they have all addressed the active site of PTPs and thus mimic substrate binding. This site comprise the phosphate binding P-loop Cys215-(X)5-Arg221 in the base of the site, extending from a β-strand (β12) going over into an α-helix (α4) (16). Several other conserved loop residues participate in substrate and inhibitor binding, including the WPD loop residues Asp181, Phg182, and residues Tyr46, Val49, Lys120, and Gln262. At the rim of the active site, Arg47 and Asp46 are located giving rise to a total depth of 8–9 Å for the site (16).

Based on the potential therapeutic usefulness of selective, non-peptide inhibitors we initiated high throughput screening of a diverse compound library using PTP1B and a synthetic 32P-phosphorylated peptide as substrate. It was our aim to identify compounds that fulfilled a set of selection criteria, which would allow further optimization. We decided to include general PTP inhibitors only. The main reason for this criteria is our wish to develop a set of inhibitors that selectively inhibit different PTPs. Therefore, the common starting point should be a general PTP inhibitor that could be optimized for selectivity and potency using structure-based approaches. To secure such a general mechanism of inhibition, the compounds should be active site-directed reversible inhibitors and show classical competitive inhibition. In particular, we wanted to avoid time-dependent inhibitors that might be difficult to optimize for specificity against different PTPs utilizing structural information for the target PTPs. Also, since it is our goal to develop inhibitors that might be orally active drug candidates, only low molecular weight compounds were included. 2-(Oxalylamino)-benzoic acid (Fig. 1, compound 1) was identified during these studies and found to be one of the most efficient phenyl phosphate mimetics identified so far.
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Experimental Procedures

Materials—p-Nitrophenyl phosphate, bovine serum albumin (BSA A-4503), catalase (C-100), NAD-dithiothreitol (DTT-D-9779), and glutathione (GSH-G-6529) were purchased from Sigma. Most other chemicals were analytical grade from Merck, Germany. Chemicals and solvents for compound synthesis were used as received.

Cloning, Expression, and Purification—cDNAs encoding the catalytic domains of PTP1B (17), SHP-1 (18), PTPe (19), PTPβ (19), and CD45 (20) were obtained by polymerase chain reaction using primers with convenient cloning sites and appropriate cDNA templates. The cDNAs were inserted into prokaryotic expression vectors as detailed in Table 1. All PPT coding sequences were confirmed by DNA sequencing. The PPT-LAR expression vector was a kind gift from M. Streuli, Boston, MA. The constructs were inserted into pGEX expression vectors (Amersham Pharmacia Biotech) and transformed into Escherichia coli BL21 (Amersham Pharmacia Biotech). Overnight cultures were diluted 1:25 and grown for 3 h at 37 °C before addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.1 mM. The glutathione S-transferase fusion proteins were purified according to the manufacturer’s instructions (Amersham Pharmacia Biotech). For x-ray crystallography, a pET11a expression plasmid encoding the first 321 amino acids of PTP1B was transformed into E. coli BL21(DE3). An overnight culture diluted 1:160 to a total volume of 2 liters of SOB medium (2% w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 10 mM NaCl, 10 mM MgCl2, and 10 mM MgSO4) and grown at 37 °C until the A600 was 0.6. Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.1 mM, and the incubation was continued at room temperature for 3 h. PTP1B was purified essentially as described previously (21).

High Throughput Screening Assay—A scintillation proximity technology based high-throughput screening assay utilizing recombinant PTP1B (17) and a biotinylated 33P-labeled peptide substrate was used to screen the Novo Nordisk compound library (to be reported elsewhere).

Determination of Inhibitor Constants, Ki—The enzyme reactions were carried out using standard conditions essentially as described by Burke et al. (22). The assay conditions were as follows. Appropriately diluted inhibitors (4 different concentrations: diluted 1: 3, 9, and 27-fold) were added to the reaction mixtures containing different concentrations of the substrate, p-nitrophenyl phosphate (usual range: 0.31 to 20 mM, final assay concentration). The buffer used was 100 mM sodium acetate, pH 5.5, 50 mM sodium chloride, 0.1% (w/v) bovine serum albumin, 5 mM glutathione, and 1 mM EDTA (total volume 100 μl). The reaction was started by addition of the enzyme and carried out in microtiter plates at 25 °C for 60 min. The reactions were stopped by addition of NaOH. The enzyme activity was determined by measuring the absorbance of the compounds and p-nitrophenol phosphate. The data were analyzed using nonlinear regression hyperbolic function. The Michaelis-Menten enzyme kinetic models. Inhibition is expressed as Ki values in micromolar.

Time Dependence—The same assay format as that described above for Ki, determinations was used. The inhibitor, compound 1, was used at different concentrations (62.5, 125, and 250 μM), whereas the concentrations of substrate, p-nitrophenyl phosphate was kept constant at 2.5 mM. The reaction was started by addition of enzyme and then stopped at 3-min intervals by addition of NaOH. The buffer used in these experiments was 100 mM sodium acetate, pH 5.5, 100 mM NaCl, 5 mM dithiothreitol, and 0.1% (w/v) bovine serum albumin with further addition of 1 mM EDTA or 25 μg/ml catalase as indicated.

Co-crystallization of PTP1B with Inhibitors—A 6–10 mg/ml preparation of PTP1B in 10 mM Tris, pH 7.5, 25 mM NaCl, 0.2 mM EDTA, and 3 mM dithiothreitol, was used for crystallization. Crystals were grown by the sitting, as well as the hanging drop, vapor diffusion methods. A 1:10 (PTP1B:inhibitor) molar ratio mixture was prepared at least 1 h prior to crystallization. Two μl of PTP1B-inhibitor solution was mixed with 2 μl of reservoir solution consisting of 0.1 M Hepes buffer, pH 7.5, 0.3–0.4 mM sodium acetate or magnesium acetate, 12–16% (w/v) polyethylene glycol 8000 and/or 4% (w/v) glycerol. The reservoir volume was 1 ml. Crystals grew to the size of 0.3–0.6 × 0.1–0.3 × 0.1–0.3 mm within 2–3 days.

Data Collection—All diffraction data collections were performed at 100 K. The following cryo conditions were used: to the hanging or sitting drop (50% (w/v) glycerol (containing 0.5 mM of inhibitor) were added. The crystal was removed from the drop after 5–30 min and transferred to 50% glycerol (containing 0.5 mmol inhibitor) and flash frozen. Data were collected using a Mar345 image plate detector either at the Max-lab synchrotron facilities at Lund University or in house on a rotating anode (RU300, CuKα) equipped with Osram multilayer mirror system. Typically a 1° oscillation per image was used for 60 images. Data sets in the resolution range 2.7–1.5 Å were obtained. The space group was determined to be P3121 for all crystals used. Data processing was performed using Denzo, Scalepack, and the CCP4 program suite (23, 24).

Refinements—As P3121 contains a polar axis and, thus, possesses more than one indexing possibility, a molecular replacement solution using Amore (24, 25) was determined prior to the refinements. A high resolution PTP1B structure was used as a starting model (PDB file: 1PTV (26)), with ligand and water molecules omitted from the structure. All refinements were performed with Xplor. version 3.851 (Molecular Simulations Inc., USA). Interchanging cycles of model building using Quanta X-build (MSI) and refinement were performed. The 2Fo – Fc maps were inspected by the use of X-ligand (MSI) at a 1.3σ level for densities that could correspond to the structures of the inhibitors. In all cases, a well suited inhibitor electron density was identified in the active site pocket (see below). No other densities were identified to fit the inhibitors. Water molecules were inserted using the X-solvent program (MSI) based on 1.5σ Fo – Fc electron density maps. For further details see Table II.

Compound Synthesis—Substituted anthranilic acids or esters were treated with ethyl oxalyl chloride in THF yielding 2-(oxalylamino)-benzoic acid mono- or diesters. Base hydrolysis of the ester group(s) gave after treatment with aqueous HCl the desired 2-(oxalylamino)-benzoic acid 1 and 5 (Fig. 1). Using the same procedure, the naphthalene analogue 2 was prepared. The indole-based compounds 3 and 4 were synthesized applying the same procedure as described for 1 using either 6-amino-H-indole-5-carboxylic acid ethyl ester or 6-amino-H-indole-7-carboxylic acid ethyl ester as starting material prepared as described by Showalter and co-workers (27). Further details regarding synthesis and SAR of this family of compounds will be published elsewhere.

Results

High Throughput Screening for General, Competitive PTP Inhibitors—Our goal was to develop selective low molecular weight PTPase inhibitors that potentially could be used for treatment of diverse disease states, such as diabetes, inflammation, and cancer. As a starting point, we therefore desired a lead compound with the following characteristics. It should (i) be a general inhibitor mimicking the binding of tyrosine phosphate (Tyr-P); (ii) be a competitive, reversible active site inhibitor; and (iii) have a molecular weight below 300 to leave room for further optimization for potency and selectivity.

Using a scintillation proximity technology-based high throughput screening assay, several inhibitors were identified, some being time-dependent inhibitors, and some were co-
### Table II

| Compound | Compound 2 | Compound 3 | Compound 5 |
|----------|------------|------------|------------|
| Space group | P3_21 | P3_21 | P3_21 | P3_21 |
| Unit cell parameters | a = b = 88.2 Å and c = 103.8 Å | a = b = 88.0 Å and c = 104.0 Å | a = b = 87.9 and c = 103.1 Å | a = b = 88.1 and c = 103.7 Å |
| Completeness (all data) | 100% (20–2.72 Å) | 88.1% (25–2.35 Å) | 99.9% (25–1.8 Å) | 99.5% (30–1.95 Å) |
| Completeness (high resolution) | 100% (2.77–2.72 Å) | 68.1% (2.39–2.35 Å) | 98% (1.83–1.80 Å) | 99% (1.98–1.95 Å) |
| Multiplicity (all data) | 6.2 (20–2.72 Å) | 3.9 (25–2.35 Å) | 4.9 (25–1.8 Å) | 2.9 (30–1.95 Å) |
| Rmerge (all data) | 11.7% (20–2.72 Å) | 8.2% (25–2.35 Å) | 6.8% (25–1.8 Å) | 5.9% (30–1.95 Å) |
| Rmerge (U) (high resolution) | 42.1% (2.77–2.72 Å) | 34.8% (2.39–2.35 Å) | 50.8% (1.83–1.80 Å) | 20.6% (1.98–1.95 Å) |
| (Iof(I)) (all data) | 10.7 (20–2.72 Å) | 10.5 (25–2.35 Å) | 15.3 (25–1.8 Å) | 11.2 (30–1.95 Å) |
| (Iof(I)) (high resolution) | 3.5 (2.77–2.72 Å) | 1.7 (2.39–2.35 Å) | 2.5 (1.83–1.80 Å) | 2.1 (1.98–1.95 Å) |
| Unique reflections | 12,852 | 17,562 | 42,767 | 34,273 |
| Atoms in structure | 2,464 | 2,555 | 2,724 | 2,693 |
| R factor* | 26.7% | 26.8% | 23.1% | 24.9% |

For a Crystallographic R factor = \( \frac{\sum |F_o| - |F_c|}{\sum |F_o|} \) Data from 6 Å to the high resolution limit were used for each data set in the R factor calculation.

\[ R_{merge} = \frac{\sum |F_o| - |F_c|}{\sum |F_o|} \text{, where T is a test set containing a random 5% of the observations omitted from the refinement.} \]

### Additional Text

Valently modifying PTPs (to be reported separately). However, in terms of fulfilling the above criteria, in particular one low molecular weight compound (M, 209) caught our attention, 2-(oxalylamino)-benzoic acid (OBA, compound 1, Fig. 1).

**2-(Oxalylamino)-Benzoic Acid** is a competitive, reversible, **Active Site Inhibitor of PTP1B**—The enzyme kinetic behavior of OBA was found to be a textbook example of a classical competitive inhibitor with a \( K_i \) value \(~20 \mu M\) against PTP1B at pH 5.5 (Fig. 2). It appears that OBA behaves as a non-cleavable tyrosine phosphate mimic. While the enzyme kinetic behavior of OBA as a classical, competitive inhibitor is fully retained at the neutral pH, the \( K_i \) value obtained shows pH dependence with a 10-fold lower potency at pH 7.0 (\( K_i = 200 \mu M\), not shown). It is of specific interest, however, that the observed affinity of OBA for PTP1B, even at neutral pH, is about 10-fold higher than the affinity of the non-hydrolyzable analog of the natural substrate, \( a, a\)-difluorobenzyl phosphonic acid (KD = 2.5 ms) (28).

**OBA** acts as a Time-independent Inhibitor—Other PTP inhibitors have been shown to be very sensitive to assay conditions such as EDTA and dithiothreitol. As an example, in a recent, thorough study it was shown that addition of EDTA or catalase prevented alendronate from inhibiting PTP1B and CD45, suggesting that a combination of alendronate, trace metal ions, and H2O2 was responsible for the observed time-dependent PTP inhibition of alendronate (14). We, therefore, analyzed if OBA inhibited PTP1B in a time-dependent manner. Fig. 3 shows that (a) there is no time dependence and (b) the inhibition is not influenced by the addition of EDTA or catalase. Taken together, our kinetic analyses clearly show that OBA acts as classical, time-independent, active site-directed, reversible competitive inhibitor that does not covalently modify PTP1B.

**OBA Can Be Optimized**—Previous studies have shown that naphthyl phosphonates are considerably more potent as PTP inhibitors than their phenyl phosphate counterparts (29). The structural basis for the increased potency of inhibitors with an additional phenyl ring has been provided by x-ray crystallography showing additional hydrophobic interactions between ligand and enzyme (22, 30). Therefore, as a first attempt to improve the potency of OBA we made compounds 2, 3, and 4 (Fig. 1). In agreement with the predictions, both the naphthyl- (2) and the indole- (3 and 4) based compounds showed increased affinity for PTP1B (Table III), although not nearly as dramatic as that observed for naphthyl phosphonate. Since part of the increased affinity most likely arises from hydrophobic interactions between the second phenyl ring of the inhibitors and hydrophobic side chains of PTP1B we also made compound 5. As shown in Table III, significant changes in the inhibitor profiles against the different PTPs are observed for these compounds. As an example, compound 3 (in comparison with OBA) shows about 30-fold increase in potency against SHP-1, but only a 2-fold increase against PTP1B. These differences are particularly noteworthy since all compounds, due to their small size, most likely address the active site binding pocket only.

**OBA Is a General PTP Inhibitor**—OBA was tested against a diverse set of catalytic domains representing 6 different PTP families. Table III shows that OBA inhibits most PTPs with PTP-LAR as a notable exception. Importantly, compound 2 shows considerable affinity for PTP-LAR, thus providing support for the notion that an aromatic carboxylic acid substituted with OBA shows about 30-fold increase in potency against PTP1B (Table III), although not nearly as dramatic as that observed for naphthyl phosphonate.

**X-ray Crystallography: Co-crystallization of PTP1B with OBA**—Although the enzyme kinetic analyses clearly indicate that OBA and compounds 2–5 act as active site inhibitors, it was impossible from these experiments to map the exact binding mode, i.e. the information needed for future structure-based design and optimization. Therefore, we undertook co-crystallization studies of PTP1B and compound 1. A well suited density was identified in the active site of PTP1B. The Fc 2Fo – Fe omit map of compound 1 in the active site is shown in Fig. 4. A and B. No other densities were identified to fit compound 1. The electron density allows an unambiguous fitting of 1 with the oxamic acid and the o-carboxy group clearly discernible.

Superimposing all equivalent atoms of PTP1B complexed with compound 1 and PTP1B C215S complexed with TytP (26), a root mean square deviation between Co atoms of 0.42 Å is observed. Hence no alterations in secondary and tertiary structure are detected. Minor side chain differences are ob-
served, and those related to the active site binding pocket will be discussed below.

The overall ligand conformation of compound 1 is almost planar with both carboxylic acid groups possessing small twists out of the plane (these torsion angle twists are consistently found for all ligand structures determined, see Figs. 4–7).

**Binding to the P-loop**—The PTP signature motif Cys215-\((X)_5\)-Arg221, the P-loop, is one excellent example of nature's design of a highly efficient binding pocket for phosphate, forming a half-circle with Cys215 almost in the center. The main chain amides are pointing toward Cys215 and together with the side chain of Arg221 they form 6 hydrogen bonds and two salt bridges with the three distal oxygens of Tyr(P) (16, 26). A similar binding pattern is observed for the PTP1B-orthovanadate complex (8).

As seen in Fig. 5, the oxalylamino part of compound 1 share several of the above Tyr(P) interaction points within the Cys215-\((X)_5\)-Arg221 motif, with the lack of hydrogen bonding to the main chain amide of Ala217 (not shown) and Gly220 as noticeable exceptions. The carboxy group of oxamic acid is positioned 2.9–3.0 Å from the guanidinium group of Arg221 forming a salt bridge, as well as a hydrogen bond with the main chain amide of Arg221 and Ser216, and the carbonyl forms a hydrogen bond with the main chain amide of Gly220. Thus, it can be concluded that compound 1 behaves as a phosphate mimetic for binding in the active site of PTP1B and probably all PTPs, in accordance with our original selection criteria.

**Movement of the WPD Loop**—Binding of Tyr(P), tyrosine-phosphorylated peptide substrates (26), bis-(para-phosphophenyl)methane (31), 1,1-difluoro-1-(2-naphthalenyl)methylphosphonic acid (22), and vanadate (8) in the active site of PTP1B, and tungstate in the *Yersinia* PTP (32) induces a dramatic conformational change in the so-called WPD loop which brings the conserved Asp181 in a position to participate in substrate binding and serve as a general acid in hydrolysis of substrates. It is believed that binding of phosphate to the conserved Arg221 in part induces the movement of the WPD loop (discussed in Ref. 30). In the PTP1B-hexapeptide complex (26), the closed WPD conformation is stabilized by hydrogen bonding between Asp181 and the phosphate group of Tyr(P) and...
by aromatic-aromatic interactions between the phenyl ring of Tyr(P) and the side chain of Phe182. A similar movement of the WPD loop is observed when compound 1 binds to PTP1B. However, in addition to the backbone motion we also observe a change in the torsion angle of Phe182 which brings the phenyl ring into a more favorable position for aromatic-aromatic interaction with the phenyl ring of 1 (see Fig. 5B). It should be noted that the aromatic cores of compound 1 and Tyr(P) are only partly overlapping (Fig. 5A).

The closure of the WPD loop brings the conserved Asp181 into an apparently unfavorable position, i.e. only 2.9 Å from the o-carboxy group of compound 1 (see Fig. 5B). To our knowledge, this is the first description of a significant conformational change of this highly conserved amino acid to accommodate a ligand. The pKₐ values for compound 1 have been determined to be 3.8 and 4.8, respectively (not shown). Thus, the o-carboxy group of compound 1 is likely to be fully deprotonated at neutral pH. This will allow the formation of a salt bridge between Lys 120 and compound 1. In addition, the o-carboxy group is within hydrogen bonding distance from the side chain of Tyr 46. Clearly the o-carboxy group of compound 1 provides additional points of interaction in the active site binding cavity as compared with Tyr(P) (Fig. 6).

The Catalytic Water Molecule—As in PTP1B complexed with peptide substrate (26), we observe a water molecule similarly positioned and trapped under the WPD loop.

**TABLE III**

| Compound | PTP1B | PTPα | PTPε | PTPβ | CD45 | PTP-LAR | SHP1 |
|----------|-------|------|-------|------|------|---------|------|
| Compound 1 | 23    | 870  | 130   | 33   | 160  | >2000   | 510  |
| Compound 2 | 9.9   | 190  | 45    | 14   | 37   | 68      | 94   |
| Compound 3 | 14    | 800  | 66    | 6.0  | 56   | 550     | 16   |
| Compound 4 | 8.0   | 190  | 33    | 3.6  | 20   | 450     | 28   |
| Compound 5 | 14    | 89   | 33    | 17   | 49   | 420     | 84   |

**FIG. 4.** 2Fₒ – Fc omit maps for the four complex structures. Contour levels are at 1s (yellow) and 3s (red). For each complex, the inhibitor has been omitted from the phasing model and refined several cycles before map calculation. Atoms are colored according to atom type (carbon in white, oxygen in red, nitrogen in blue, and iodine in green). The eight pictures show the inhibitors in omit maps and the location of these in the active site pocket of PTP1B, respectively. A and B displays compound 1; C and D compound 2; E and F compound 3; and G and H compound 5.
shows a consistent binding mode, both regarding ligands and protein (see Fig. 7). Identical hydrogen bonding patterns are seen for all of the complexed structures. The phenyl ring of [1,1-difluoro-1-(2-naphthalenyl)methyl]phosphonic acid (22) and the second phenyl ring of compound 2 almost share binding mode and interact with several non-polar residues, including van der Waals contacts with Ala217, Ile219, Val49, and aromatic-aromatic interaction with Tyr46. These hydrophobic interactions are also present for the iodine of compound 5 and in part for compound 3.

**DISCUSSION**

Several attempts to make PTP inhibitors by replacing the phosphate functionality of Tyr(P) containing peptide substrates with non-hydrolyzable phosphate mimetics have been reported. Initial experiments included sulftyrolyl (34–38), phosphonates (15, 39), and O-malonyltyrosine (40, 41). In most studies, the Tyr(P) mimetics were incorporated in peptides known to be substrates for PTPases. In particular, one peptide derived from the epidermal growth factor receptor, EGF-R922–935 (Ac-DADEXL-NH₂, where X denotes Tyr(P) or Tyr(P)
When difluorophosphonomethyl phenylalanine (F2Pmp) is introduced in the EGF-R922–993 peptide, a potent inhibitor of PTP1B is obtained with \( \text{Ki} = 180 \text{ nM} \) (15). Since the “minimal unit” of F2Pmp, \( \alpha, \alpha \)-difluorobenzyl phosphonic acid, shows a \( \text{Ki} \) of 2.5 mM for PTP1B (28), this suggests that the peptide part of the inhibitor contributes significantly to the overall binding affinity. Incorporation of O-malonyltyrosine and fluoro-O-malonyltyrosine in the Ac-DA-DEXL-NH2 peptide leads to PTP1B inhibitors with IC50 values of 10 and 1 mM, respectively (42, 43). It is difficult to make a direct comparison of OBA with the above phenyl phosphate/Tyr(P) mimetics. Nonetheless, given the fact that OBA at neutral pH (without being incorporated in a peptide) displays a \( \text{Ki} \) of 200 mM for PTP1B would indicate that this compound is one of the most potent minimal unit active site PTP1B inhibitors identified so far.

The \( \alpha \)-carboxylic acid of OBA occupies a different part of the active site when compared with the Tyr(P) substrate. A unique arrangement is thus formed with a salt bridge connecting the \( \alpha \)-carboxylic acid and Lys 120. Furthermore, the \( \alpha \)-carboxylic acid is in hydrogen bond distance with Tyr 46 and Asp181. This arrangement is critical for the observed affinity for PTP1B since removal of the \( \alpha \)-carboxy group from compound 1 increases the \( \text{Ki} \) value to 2000 mM (not shown). In this context, it is of interest that Burke and co-workers (41) recently showed that introduction of a \( \alpha \)-carboxy group into 4-(O-carboxymethyl)-L-tyrosine increased the potency more than 100-fold when incorporated in the above Ac-DADEXL-NH2 peptide.

Only one x-ray structure of PTP1B in complex with a non-phosphonate PTP inhibitor, a fluoromalonyl tyrosine-based cyclic peptide, has been reported (30). Although the fluoromalonyl tyrosine peptide binds to the PTP signature motif of the active site of PTP1B, this was not accompanied by closure of the WPD loop. In contrast, we found that the WPD loop closes in all structures reported here. This motion brings (i) the side chain of Phe182 into a favorable position for aromatic-aromatic interaction with OBA and its derivatives, (ii) the side chain of Asp181 close to the \( \alpha \)-carboxylic acid of OBA. The optimal positioning of both of the carboxy groups of OBA is likely to contribute significantly to the potency of this class of compounds.

When comparing with the complexes of PTP1B and Tyr(P) containing peptides (26), several structural differences have been identified in the present study. Thus, in addition to the above movement of Lys120, a minor rotation of the Phe 182 side chain is observed (Fig. 5B), most likely due to the shift seen in the aromatic cores between OBA and Tyr(P) (Fig. 5A). Similarly, the different orientation of the Asp181 side chain might be due partly to the shift in the aromatic core, partly to interaction with the \( \alpha \)-carboxylic acid group. This demonstrates that the active site of PTP1B, although fairly rigid, possesses the capacity to accommodate other ligands that have no or limited similarity to the natural substrate. This accommodation process is observed in the surface exposed loop regions, whereas no differences in the PTP loop are observed between the Tyr(P) and OBA complex structures. As indicated above, the potency of OBA for PTP1B (and possibly most other PTPs) appears to arise from (i) OBA’s ability to closely mimic the Tyr(P) substrate (phosphate and aromatic ring) and (ii) as well as the formation of a novel set of contacts with residues Asp181, Lys120, and Tyr-46.

The general interest in phosphate recognition sites as drug targets (e.g. in serine and tyrosine phosphatases, SH2 domains,
PTB domains, and also kinases) points to the importance of identification of novel biologically active phosphate mimetics. Compounds containing one or more carboxylic acids have been particularly useful in the design of PTP inhibitors (6). However, although significant progress has been made in this area, the current study emphasizes the need for correctly positioning the carboxylic acid-based PTP inhibitors. Interestingly, Beaulieu and co-workers (44) recently reported the replacement of phosphate with the non-hydrolyzable oxamic acid group in peptide-based SH2 domain ligands. Although 20 times less potent than phosphate, these findings add to the notion of oxamic acid as a phosphate mimic.

In conclusion, we have demonstrated that OBA is a (i) novel, (ii) general, (iii) low molecular weight, (iv) classical, competitive, and (v) active site inhibitor of PTPs. Detailed enzyme kinetic analyses in combination with extensive x-ray crystallographic evaluation of OBA and three derivatives have shown that this class of inhibitors, in addition to utilizing many of the interaction points of the natural substrate Tyr(P), also create a unique binding mode. We anticipate that OBA will be an essential tool in future explorations of the structure and function of PTPs. Furthermore, the structural insight provided here should make OBA an ideal starting point in our search for potent and selective PTP inhibitors.

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