Structure-based Design of Selective and Potent Inhibitors of Protein-tyrosine Phosphatase β*

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Protein-tyrosine phosphatases (PTPs) are considered important therapeutic targets because of their pivotal role as regulators of signal transduction and thus their implication in several human diseases such as diabetes, cancer, and autoimmunity. In particular, PTP1B has been the focus of many academic and industrial laboratories because it was found to be an important negative regulator of insulin and leptin signaling, and hence a potential therapeutic target in diabetes and obesity. As a result, significant progress has been achieved in the design of highly selective and potent PTP1B inhibitors. In contrast, little attention has been given to other potential drug targets within the PTP family. Guided by x-ray crystallography, molecular modeling, and enzyme kinetic analyses with wild type and mutant PTPs, we describe the development of a general, low molecular weight, non-peptidic, non-phosphorus PTP inhibitor into an inhibitor that displays more than 100-fold selectivity for PTPβ over PTP1B. Of note, our structure-based design principle, which is based on extensive bioinformatics analyses of the PTP family, are general in nature. Therefore, we anticipate that this strategy, here applied to PTPβ, in principle can be used in the design and development of selective inhibitors of many, if not most PTPs.

Protein-tyrosine phosphatases (PTPs) are key regulators of signal transduction. Together with the counteracting protein-tyrosine kinases, they control the phosphorylation status of many important proteins and are thereby critically involved in the regulation of fundamental cellular processes such as metabolism, cell growth, and differentiation. Aberrant tyrosine phosphorylation levels have been associated with the development of cancer, autoimmunity, and diabetes, thus indicating that PTPs might play important etiological and pathogenic roles in these diseases (1–5). In particular, two elegant studies that PTPs might play important etiological and pathogenic implications in Refs. 3, 13, and 14), and several research groups have successfully used structure-based designs to synthesize active site-directed, selective PTP1B inhibitors (15–20). Most important, two groups have demonstrated recently that it is possible to develop compounds that are selective for PTP1B over the highly homologous T cell-PTP (21, 22), thereby lending support to the view that selective inhibitors, which discriminate between even closely related PTPs, are within reach.

In contrast, and despite the increasing amount of data indicating that other PTPs, including CD45, LAR (leukocyte antigen-related), PTPα, and SHP-1, could also be considered as attractive therapeutic targets, only little attention has been given to the design of compounds that selectively inhibit these enzymes (reviewed in Refs. 3, 5, 12, and 23). Of note, when searching for PTP inhibitors, there is a significant risk of identifying oxidizing or alkylating compounds, i.e. compounds that are not true inhibitors. Indeed, when searching for PTP inhibitors, it is a common experience among laboratories that perform high throughput screenings to get high hit rates, which in many cases are caused by oxidation or alkyla tion of the active site cysteine (16, 24). Although compounds that irreversibly and/or more broadly in a time-dependent manner modify PTPs may still be useful in the clinic (25–27), structure-based optimization is often difficult, if not impossible. Hence, there is a need for development of tools that will allow structure-based design of selective inhibitors of other members of the PTP family.

The objective of the present study was to investigate if a general, low affinity, and active site-directed PTP inhibitor, which was previously optimized for PTP1B selectivity, could be used as a synthetic starting point for the design of compounds that selectively inhibit other PTPs but not PTP1B. In fact, our ultimate goal is to develop a general technological platform that will allow synthesis of selective inhibitors of most and possibly all classical PTPs. The design strategy is shown schematically in Fig. 1, and it is based on the development of inhibitors that simultaneously address the active site and defined selectivity determining residues in the vicinity of the

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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; OATP, 2-(oxalylamino)-4,7-dihydro-5H-thieno-[2,3-c]pyran-3-carboxylic acid; pNPP, p-nitrophenyl phosphate; wt, wild type; DTT, dithiothreitol.

2 C. B. Jeppesen, S. Branner, and N. P. H. Møller, unpublished observations.
active site pocket. First, by using high throughput screening, we identified 2-(oxalyl-amino)benzoic acid (OBA) (compound 1, Fig. 2) as a general, active site-directed PTP inhibitor scaffold that binds to most PTPs but with low affinity (i.e. compound A). Selectivity is obtained by addition of substituents (i.e. b or c) that fit in one subset of PTPs, but not in other PTPs, by addressing residues or combinations of residues unique to the PTP in question and positioned in the vicinity of the active site. Additional substituents are used to increase both the potency and selectivity (d and e).

To validate the general applicability of our approach, we decided to investigate if the 47-48-258-259 region, which is open cleft in PTP1B and thus allows for direct access to synthetic scaffold and by addressing the 258 non-phosphorous PTP inhibitor is used as starting point (compound A). Selectivity is obtained by addition of substituents (i.e. b or c) that fit in one subset of PTPs, but not in other PTPs, by addressing residues or combinations of residues unique to the PTP in question and positioned in the vicinity of the active site. Additional substituents are used to increase both the potency and selectivity (d and e).

**EXPERIMENTAL PROCEDURES**

**Materials**

-p-Nitrophenyl phosphate (pNPP) and DL-dithiothreitol (DTT, D-9779) were purchased from Sigma. Most other chemicals were analytic grade from Merck. Water was purified in a Millipore purification system (18 megohms/cm; Millipore Inc.). Glutathione-Sepharose, Sephadex G-25, Q-Sepharose Fast Flow, Mono Q, and Superdex 200 were from Amersham Biosciences.

**Cloning, Expression, and Purification**

Cloning of the catalytic domains of PTP1B, PTPβ, GLEPP1 (residues 885-1138), PTPs domain 1, and CD45 domain 1-2 were performed as described previously (28, 30). The PTP LAR expression vector was a kind gift from M. Streuli, Boston. The following PTP mutants were made by overlap extension PCR using appropriate restriction sites for cloning purposes: PTP1B to PTPβ: (i) R47N and D48N; (ii) D48N; PTPβ to PTP1B: N47R and N48D. All constructs were inserted into pGEX expression vectors (Amersham Biosciences). For x-ray protein crystallography, the cDNA encoding the first 321 amino acids of PTP1B was inserted into the pET11a expression vector. All coding sequences were confirmed by DNA sequencing. Expression and purification of the glutathione S-transferase fusion proteins (wild type and mutant) and the protein for x-ray protein crystallography were performed as described previously (28).

**Determination of Inhibitor Constants, K<sub>i</sub>**

The phosphatase activity was assayed using pNPP as substrate and carried out essentially as described previously (30, 37). The assay buffer (pH 7.0), consisting of 50 mM 3,3-dimethylglutarate, 1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100, was adjusted to an ionic strength of 0.15 M by addition of NaCl. In brief, appropriately diluted inhibitors (undiluted and three times 3-fold dilutions) were added to the reactions mixture containing various concentrations of pNPP (range of 0.32–20 mM, final assay concentration, total volume of 100 μl). The reaction was initiated by addition of the enzyme, carried out at 25 °C for 30 min, and stopped by addition of 0.5 M NaOH in 50% ethanol. The enzyme activity was determined by measuring the absorbance at 405 nm using a Spectra MAX384 microplate spectrophotometer (Molecular Devices) with appropriate corrections for absorbance of substrate, compounds, and nonenzymatic hydrolysis of substrate. The data were analyzed using a nonlinear regression hyperbolic fit to classical Michaelis-Menten enzyme kinetic models. Inhibition is expressed as K<sub>i</sub> in μM. The reported standard deviations values are calculated from at least three independent experiments.

**Crystallization**

For co-crystallization of PTP1B with compound 5, a 10 mg/ml PTP1B solution in 10 mM Tris, pH 7.5, 25 mM NaCl, 0.2 mM EDTA, and 3 mM DTT was used. A 1:10 (PTP1B/compound 5) molar ratio mixture was prepared at least 1 h prior to crystallization. Crystallization experiments were conducted by the hanging drop vapor diffusion method. 2 μl of the PTP1B/compound 5 solution was mixed with 2 μl of reservoir solution consisting of 0.1 M Hepes buffer, pH 7.5, 0.3–0.4 M sodium acetate or magnesium acetate, 12–16% (w/v) polyethylene glycol 8000,
Selective Inhibitors of PTPβ

Table I
Statistics of x-ray crystallographic data and structure refinements

| Ligand     | PTP1B | Compound 8 |
|------------|-------|------------|
| Space group| P3121 | P3121      |
| Cell parameters (Å) | a = b = 88.136 | a = b = 88.192 |
| Resolution range (Å) | 20–2.35 | 50–2.25 |
| Unique reflections | 19,322 | 22,597 |
| Multiplicity | 5.9 | 5.1 |
| Completeness (%) | 96.7 (98.0) | 99.9 (99.7) |
| Rmerge (%) | 11.4 (41.7) | 9.2 (48.3) |
| Rfree (%) | 12.1 (2.8) | 12.8 (2.3) |
| R-factor (S/R-form of ligand) | 21.1/21.2 | 20.1/18.8 |
| R-free (S/R-form of ligand) | 23.7/23.5 | 23.9/23.4 |
| Root mean square deviation | 0.006/0.006 | 0.005/0.005 |
| Bond lengths (Å) (S/R-form of ligand) | 1.29/1.31 | 1.18/1.20 |

* The values in parentheses correspond to the outermost resolution shell.
* R-factors were calculated using all data. Crystallographic R-factor = Σ|||Fo||| – |Fcalc|/Σ|Fo|.
* R-free = Σ|Fo(hkl)| – |Fcalc(hkl)|/Σ|Fo(hkl)|, where T is a test set containing a random 5% of the observations omitted from the refinement process.
* Refinements of PTP1B in complex with the S- and R-enantiomeric forms of the ligand, respectively.

and/or 4% 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. For crystallization of PTP1B with compound 8, a 20 mg/ml PTP1B solution in 10 mM Tris, pH 7.5, 25 mM NaCl, 0.2 mM EDTA, and 3 mM DTT was used. A 1:10 (PTP1B/compound 8) molar ratio mixture was prepared at least 1 h prior to crystallization. Hinging drops containing 1 µl of the PTP1B/compound 8 solution and 1 µl of reservoir solution (0.1 M Hepes, pH 7.5, 0.2 mM magnesium acetate, 10–13% (v/v) polyethylene glycol 8000, and 0.1% (v/v) β-mercaptoethanol) were equilibrated overnight. Thereafter, crystals were grown by micro-seeding with stock seed solutions of PTP1B nuclei (obtained from crystals of PTP1B in complex with compound 3 (19)) at room temperature. Crystals of data collection quality appeared within 2–3 days.

Data Collection
Data collections were performed at 100 K and with one crystal only per data set. The following cryo-condition was used: the hanging drop, 3.0 (0.5 mmol of compound 5) or 1.5 µl (1.5 mmol of compound 8) of 50% (v/v) glycerol were added. The crystal was removed from the drop after 5–30 min (compound 5) or immediately (compound 8), transferred to 50% glycerol (containing 0.5 mmol of compound 5 or 1.5 mmol of compound 8), and flash-frozen in a stream of nitrogen. For compound 5, data were collected using a Mar345 image plate detector at the MAXlab synchrotron facilities at Lund University. For compound 8, data were collected using a Mar345 image plate detector on a rotating anode (RU300, CuKα, 50 kV/100 mA) equipped with Osmic multiplier mirror system. A 0.5° oscillation per image was used for 200 images for both data sets. Data were processed using Denzo, Scalepack (38), and the CCP4 program suite (39). The space group was determined to be P3121 for both data sets.

Structure Determination and Refinements
As P31,21 contains a polar axis and, thus, possesses more than one indexing possibility, a molecular replacement solution using Amore (40) was determined prior to the refinements. The coordinates of high resolution structures of PTP1B (Protein Data Bank code 1PTV for compound 5 and Protein Data Bank code 1C88 for compound 8) were used as search models (water molecules and ligands were omitted from the structures). All refinements were performed with CNS (Accelrys Inc.). Interchanging cycles of model building using Quanta X-build (Accelrys Inc.) and refinement were performed. Water molecules were inserted using the X-solvate program (Accelrys Inc.) based on 3σ |Fo| – |Fcalc| density maps. The crystallographic data and statistics of structure refinement are given in Table I.

Molecular Modeling
Conformational Analysis—The molecular mechanics package MacroModel (41) was used to evaluate the conformational energy of the bound ligand and structure relative to the global energy minimum or other local minima. The force field MMFF, as implemented in MacroModel, was employed with default settings. All calculations were run on an SGI challenge computer. First, the conformational space was searched using the Monte Carlo multiple method (42). The energy minimizations were carried out utilizing the truncated conjugate algorithm using a model for aqueous solution (i.e., a generalized Born, solvent-accessible surface area, continuum dielectric solution model) (43). The conformational searches were continued until all low energy minima had been found multiple times. Second, the energy of the ligand in the conformation observed in the x-ray structure was estimated. The crystallographic structure was partially relaxed in the force field. A set of heavy atoms was tethered to the crystallographic positions by harmonic flat-bottomed Cartesian constraints. The flat-bottomed radius was 0.3 Å, i.e., the distance each atom is allowed to move from the tether position before an energy penalty is applied. At larger distances, a harmonic penalty function with a force constant of 500 kJ/mol Å² was applied. The two carboxylic acid groups of compound 5 were omitted in the calculations because no structural variations have ever been observed in these parts of the inhibitors (19, 30).

FlexX Docking—In this study, we applied the FlexX docking program (44), which is a common docking program used for high throughput docking applications. FlexX uses an efficient incremental construction method (45) to optimize the interaction between a flexible ligand and a rigid binding site. In this methodology, an empirically derived scoring function, which is optimized to reproduce experimental binding affinities and binding conformations for various crystallographic resolved protein–ligand complexes, is used to predict the free energy of binding (ΔGbind). In all docking experiments presented here, a scoring function with default parameters was used. Formal charges were assigned to the ligands. The pKa of the oxalamide is 2–2.5, whereas the pKa of the other carbamate is close to 4 in compounds 1–3. Therefore, the oxygens of these carbamates were assigned a charge of –1/2. Asp-48, Lys-120, and Asp-181 were ionized in PTP1B, whereas only Asp-181 and Lys-120 were ionized in PTPβ.

PTPβ Homology Building
Based on an alignment of the sequences of PTP1B and PTPβ and by the use of the program Modeler (46), a model of PTPβ at the atomic level was built. The x-ray structure of PTP1B co-crystallized with a small molecule inhibitor (Protein Data Bank code 1c87) was used as a template in homology modeling. Special attention was devoted to the active site region.

Compound Synthesis
2-Hydroxymethyl-tetrahydro-pyran-4-one (used as starting material) was obtained via catalytic hydrogenation of 2-benzoxymethyl-2,3-dihydro-tetrahydro-pyran-4-one (47) in ethyl acetate. By using the reaction conditions for synthesis of 2-aminothiophenyl (48), 2-hydroxymethyltetrahydro-pyran-4-one was reacted with tert-butyl cyanacetic acid affording a regioisomer mixture of 2-amino-5- or 7-hydroxymethyl-4,7-dihydro-5H-thieno[2,3-c]pyran-3-carboxylic acid tert-butyl ester. Silification of the hydroxyl group with triethylsilylechloride afforded a separable (column chromatography, silica gel) mixture from which the 7-regioisomer was collected. Oxalation of the amino group with imidazol-1-yl-oxyacetic acid tert-butyl ester (49) in tetrahydrofurran followed by de-silylation with 0.1 N HCl gave the key intermediate 2-((tert-butoxycarbonylamino)-7-hydroxymethyl-4,7-dihydro-5H-thieno[2,3-c]pyran-3-carboxylic acid.
RESULTS

Selection of Synthetic Scaffold—The low molecular weight, non-phosphorous, and general PTP inhibitor OATP (compound 4, Fig. 2) was selected as a synthetic starting point for the following reasons. (i) It is a competitive and active site-directed PTP inhibitor that shares many interaction points with Tyr(P) (28). (ii) Its pyran oxygen atom promotes attraction to Asn-48-PTP inhibitor that shares many interaction points with Tyr(P) (Fig. 2) with a phthalimide methyl amide and the oxygen atom of OATP-based inhibitors, i.e. the oxalyl-amide and the o-carboxyl groups, interacted as reported previously (19) with the P-loop and Lys-120. As expected, the side chain of Asp-48 was found in the rotamer 1 position, i.e. pointing away from the active site because of the repulsion exerted by the inhibitor pyran oxygen atom (30). Most significant, the inhibitor phthalimide side chain is positioned in the vicinity of the selectivity determining region constituted by Arg-47 and Arg-48, leading to a number of important interactions that provide a structural explanation for the observed increased affinity for PTP1B (Fig. 4). Noteworthy are three hydrogen bonding interactions, i.e. between the phthalimide OH group and the main chain carbonyl of Arg-45 and the previously described water-mediated interaction between the pyran oxygen and the carboxylate group of Asp-48 (Fig. 4) (30).

Molecular Modeling, Low Energy Conformation of Compound 5 in PTPβ—Could it be that the phthalimide substituent of compound 5 in PTPβ binds to the backbone amides rather than to the side chains of Asn-47 and Asn-48, similar to the situation observed in PTP1B and in contrast to our predictions? Because we have not been successful in obtaining the structure of PTPβ co-crystallized with compound 5, we turned to molecular modeling with the objective of formulating a working hypothesis that could be tested experimentally using reciprocal mutational analyses (see below). To test the validity of the applied modeling approach, compound 5 was docked into

![Chemical structures](image)

**Fig. 2. Chemical structures.**

**Table II**

| Compound | Ki (μM) for wt PTPβ | Ki (μM) for wt PTP1B |
|----------|---------------------|----------------------|
| 4        | 0.61 ± 0.04         | 0.49 ± 0.04          |
| 5        | 0.61 ± 0.04         | 0.49 ± 0.04          |
| 6        | 0.61 ± 0.04         | 0.49 ± 0.04          |
| 7        | 0.61 ± 0.04         | 0.49 ± 0.04          |
| 8        | 0.61 ± 0.04         | 0.49 ± 0.04          |
| 9        | 0.61 ± 0.04         | 0.49 ± 0.04          |
| 10       | 0.61 ± 0.04         | 0.49 ± 0.04          |

*All measurements were made using pNPP as a substrate at pH 7.0, ionic strength of 0.15 M, and at 25 °C.*
PTP1B with Asp-48 in the rotamer 1 position. As shown in Fig. 5A, the modeled (gray and magenta colored structures) and the x-ray crystallographic (green) structures have almost identical conformations.

By using the same approach as above, a completely different conformation of compound 5 was observed, when this inhibitor was docked into a homology built model of PTP\textsubscript{/H9252} (Fig. 5B). In contrast to our observations from the PTP1B-compound 5 complex (Fig. 5A), the PTP\textsubscript{/H9252}-compound 5 modeled structure indicates that the preferred interactions in PTP\textsubscript{/H9252} are in fact to the side chains of Asn-47 and Asn-48. Fig. 5B illustrates two different conformations of compound 5 bound to PTP\textsubscript{/H9252} as determined by FlexX (Fig. 5A, conformation A, gray) and by MacroModel (Fig. 5A, conformation B, magenta). The latter model constitutes the lowest obtainable energy conformation with a global minimum of 32.5 kJ/mol. Because of a steric clash with Asn-47, the docked structure (conformation B) is forced to attain a conformation with close contact between the pyran oxygen and the carbonyl group in the phthalimide ring (i.e. conformation A, Fig. 5C), possessing an energy of 39.7 kJ/mol as determined by an energy minimization and constraining by flat-bottom potential of this conformation. Thus, a significant energy penalty of 7.2 kJ/mol is observed because of the steric clash with Asn-47, which, in accordance with a recent survey of 322 unrelated proteins (51), was built to attain a rotamer position that allows formation of an internal hydrogen bond between the side chain carbonyl oxygen and the backbone nitrogen at residue 48 in PTP\textsubscript{β}. This prevents the phthalimide oxygen (carbonyl in position 1) from hydrogen bonding as effectively as it does in PTP1B.

The predicted binding mode of compound 5 shows that the phthalimide side chain may form important interactions with the side chains of Asn-47 and Asn-48 (Fig. 5, B and C). However, we have observed previously significant ligand-induced conformational changes of both the side chains and main chain in PTP1B with other types of inhibitors (not shown (30, 37)). It should therefore be emphasized that only a slight movement of the side chain of Asn-47 in PTP\textsubscript{β}, i.e. a different rotamer position, would leave room for the low energy conformation of compound 5 (conformation B in Fig. 5B) and moreover allow hydrogen bond formation between the side chain of Asn-47 and the phthalimide carbonyl of compound 5. This could potentially decrease the above close contact between the inhibitor pyran oxygen and carbonyl group and lead to an increased stabilization of the conformation because of the additional hydrogen bond formation.

Taken together, x-ray crystallography analysis and molecular modeling provide strong support for the notion that the observed identical affinities of compound 5 for PTP\textsubscript{β} and PTP1B are due to completely different binding modes.

The Presence and Position of the Phthalimide OH Group Is Critical for PTP1B Affinity—As shown above, a hydrogen bond between the phthalimide OH group of compound 5 and the main chain nitrogen of Arg-47 in PTP\textsubscript{β} was identified as a putative important contributor to the observed increased binding affinity of compound 5 compared with that of OATP (Fig. 4). To investigate the significance of this OH group, the affinities of compounds 6 and 7 (Fig. 2) were analyzed. Table II shows that the exchange of the phthalimide OH group with a fluorine or the movement of the OH group to position 5 in both cases gave rise to a dramatic decrease in affinity for PTP1B, indicating that the phthalimide OH group in position 4 plays an important role for the gain in affinity by optimal positioning of the phthalimide side chain. In contrast, the affinities of compounds 6 and 7 for PTP\textsubscript{β} were similar to that of compound 5 (Table II).

Removal of the Phthalimide OH Group Leads to Selectivity for PTP\textsubscript{β}—Based on the above experiments, we reasoned that...
a removal of the OH group from the phthalimide side chain would cause a significant selectivity improvement in favor of PTP\(_{\beta}\), due to a loss of affinity for PTP1B. Indeed, a dramatic decrease in PTP1B affinity was observed with compound 8 (Fig. 2) compared with compound 5. In contrast and as predicted, the potency toward PTP\(_{\beta}\) is almost retained with only an ~2-fold reduction in affinity (Table II). Noticeably, compound 8 displays the characteristics of a classic competitive inhibitor (not shown). In addition, this inhibitor exhibits almost the same \(K_i\) value as compound 4 for PTP1B, thus indicating that compound 8 most likely attains a conformation similar to conformation A with little or no interaction with Arg-47 and Asp-48 (Fig. 5B).

To investigate this result at the structural level, the crystal structure of the PTP1B-compound 8 complex was determined. An electron density, fitting “the OATP part” of compound 8 (not shown), was found in the active site pocket with a similar binding pattern as observed with compound 5. In contrast and as predicted, the potency toward PTP\(_{\beta}\) is almost retained with only an ~2-fold reduction in affinity (Table II). Noticeably, compound 8 displays the characteristics of a classic competitive inhibitor (not shown). In addition, this inhibitor exhibits almost the same \(K_i\) value as compound 4 for PTP1B, thus indicating that compound 8 most likely attains a conformation similar to conformation A with little or no interaction with Arg-47 and Asp-48 (Fig. 5B).

Residue 48 Is Critical for High Affinity Binding of Phthalimide-based Compounds in PTP\(_{\beta}\)—Whereas the binding modes of compounds 5 and 8 were determined in PTP1B using x-ray crystallographic analyses, the proposed binding mode in PTP\(_{\beta}\) relied on molecular modeling. Previously, we successfully used reciprocal mutational analyses to determine the specific interaction points of inhibitors in PTPs (19, 30). To unequivocally determine the binding mode in PTP\(_{\beta}\), we decided to change PTP1B into a PTP\(_{\beta}\)-like enzyme in the selectivity determining region by mutating Arg-47 and Asp-48 to the corresponding residues in PTP\(_{\beta}\). In agreement with previous findings, the parent compound (OATP, compound 4) (19) showed increased...
affinity for this mutant (PTP1B<sub>R47N,D48N</sub>) compared with wt PTP1B and wt PTPβ (Table III), thus reflecting an interaction between the pyran oxygen and the side chain of Asn-48. According to our hypothesis and the findings with wt PTPβ, the PTP1B<sub>R47N,D48N</sub> mutant should bind OATP derivatives possessing phthalimide side chains with high affinity. Indeed, compounds 5–8 have similar affinity for this mutant and for wt PTPβ. Conversely, by mutating PTPβ into a PTP1B-like en-
zyme in the 47–48 region (i.e. PTPβ<sub>R47N</sub>,<sub>D48N</sub>), a dramatic drop in affinity was observed for compounds 6–8. Of note, compound 5 still retained considerable affinity for this mutant, most likely reflecting dual interaction with the main chain nitrogens similar to the observations in wt PTP1B (Table III).

These results provide evidence for the notion that the phthalimide-based compounds bind to the side chains of residues 47 and 48 in PTPβ. To distinguish between the importance of these residues in inhibitor binding, we finally produced a single point mutated PTPβ<sub>β</sub>-like enzyme by introducing an asparagine in position 48 of PTP1B. With the exception of compound 8, all compounds showed similar affinities for PTP1B<sub>βR47N</sub> and PTP1B<sub>R47N,D48N</sub> (Table III), thus demonstrating that the major interaction point is Asn-48.

**Enantiomeric Selectivity for PTPβ**—Compounds 5–8 are racemic mixtures. Ideally, enantiopure compounds should have been used for the above analysis. However, the limited amounts of several compounds and the complicated purification procedure did not allow separation of all compounds. Clearly, our analyses with the racemic compounds have provided reliable results that could be used for structure-based design. However, we decided to produce sufficient amounts of compound 8 to allow separation of the two enantiomers (i.e. compound 9 (S) and 10 (R)). Compound 9 (Fig. 2) displayed a 2–3-fold increase in affinity for PTPβ and the PTPβ-like mutants in comparison with compound 8, whereas a significant reduction in potency was observed with the R-enantiomer toward these enzymes (compound 10, Fig. 2; Table IV). In contrast and similarly to compound 8, both compounds 9 and 10 show low binding affinity for PTP1B and the PTP1B-like mutant (Table IV). X-ray crystallographic evaluation of the two enantiomers in complex with PTP1B revealed a more well-defined electron density of the S-form than the R-form, resulting from a more stable conformation of the compound in relation to the pyran- and water-mediated interaction with the side chain of Asp-48 in PTP1B (not shown). Of note, this possibility for interaction does not exist with the R-form of compound 8, because the angle as well as the distance between the inhibitor pyran oxygen and the water molecule is inadequate for such an interaction. We hypothesized that the same structural explanation applied to the PTPβ-compound 8 complex, and we concluded that the lower potency of the R-enantiomer primarily was because of this changed conformation of the pyran ring, resulting both in the loss of a putative water-mediated interaction with Asn-48 and a less optimal presentation of the phthalimide side chain.

**Selectivity of Phthalimide-based Inhibitors**—By having demonstrated that OATP can be used as a synthetic scaffold for the design of compounds that are highly selective for PTPβ over PTP1B, we finally analyzed if the phthalimide-based compounds were also selective for PTPβ over other PTPs. Although limited availability of these compounds prevented us from testing at very high concentrations and hence from exact determination of the <i>K<sub>i</sub></i> values, the results presented in Table V demonstrate that the phthalimide side chains do not lead to high affinity against a set of different PTPs, with GLEPP1 as the only noticeable exception. Most important, even PTPs with an asparagine in position 48 (e.g. PTPs and LAR) do not bind these compounds with appreciable affinity.

In contrast, GLEPP1 is significantly inhibited by all compounds with the exception of OATP (compound 4) and compound 10, which only displayed a moderate inhibition. Despite this relatively high affinity of the inhibitors toward GLEPP1, a 6–14-fold difference in potency between PTPβ and GLEPP1 was still preserved. Because GLEPP1 belongs to the same subfamily as PTPβ, and therefore most likely possesses similar structural features, these findings were not unexpected. Clearly, more work is required to obtain highly selective inhibitors of either PTPβ or GLEPP1.

**DISCUSSION**

The objective of the present study was to investigate if a general and active site-directed PTP inhibitor, which has been used previously for optimization of selectivity for PTP1B, could be utilized as a synthetic starting point for the design of selective inhibitors of other PTPs with simultaneous loss of activity against PTP1B.

With the completion of the Human Genome Project, we now have a comprehensive overview of the family of classical PTPs, which consists of a total of 38 functional genes (13). Initially, the expectations to the total number of PTPs were considerably higher (52, 53), and similar to the situation in the kinase field, it was considered extremely difficult, if not impossible, to develop highly selective PTP inhibitors that address the active site. The potential problems in identifying active site-directed inhibitors were further highlighted when early structural studies revealed that even distantly related PTPs, such as PTP1B and the Yersinia PTP, have similar three-dimensional structure and contain the same invariant residues (54, 55). However, careful bioinformatics and structural analyses revealed that combinations of residues in the vicinity of the active site potentially could be used as selectivity determining regions and hence be addressed by active site-directed inhibitors (14, 29). Thus, in contrast to the initial concerns, it now seems that the highly conserved nature of the catalytic domains of PTPs represents an advantage and excellent opportunity for structure-
based design of highly selective inhibitors of this class of enzymes. Furthermore, with the limited number of PTP genes it is not an insurmountable task to clone and express all PTP domains, thus allowing detailed analyses of inhibitor specificity at the biochemical level.

After the initial reluctance to enter the PTP inhibitor field, a number of laboratories in academia and the pharmaceutical industry have actively pursued structure-based design of selective compounds. These endeavors have been strongly supported by the availability of an impressive number of x-ray structures (reviewed in Ref. 56). Furthermore, the seminal publications that demonstrated that PTP1B is a key negative regulator of insulin and leptin signaling (6–9) provided the first evidence that PTPs indeed could be attractive molecular targets. As a result, very significant progress was quickly achieved in the development of highly selective and potent inhibitors of PTP1B (11, 15–22, 30, 57, 58).

In addition to PTP1B, several other PTPs could also be attractive drug targets (32, 33). However, up to this point few research groups have reported selective inhibitors of other PTPs. In this context it should also be mentioned that the very nature of the PTP active site with a catalytically essential cysteine, which is susceptible to redox regulation, may confound the evaluation of potential lead structures. Thus, it is now becoming clear that the high hit rates, which are often observed when screening for PTP inhibitors, in many cases are due to oxidation or alklylation of the active site cysteine rather than to true inhibition (16, 24). To avoid such problems and to create a general technological platform, we have here explored the possibility of utilizing a general, low molecular weight, active site-directed PTP inhibitor as a synthetic scaffold for the design of inhibitors that are selective for other PTPs than PTP1B. Most important, the key elements of this inhibitor were utilized previously (19, 30) to design selective PTP1B inhibitors. We have selected PTPβ as a model enzyme for these studies. Although the exact physiological role of this PTP remains to be defined, it seems to represent a PTP subfamily, which appears to be critically involved in regulation of cell growth and motility and perhaps the development of cancer (34–36). However, despite the fact that DEP-1 and PTPβ show a high degree of sequence identity in the above-mentioned selectivity determining region (29), they seem to have quite different substrate specificity. Thus, whereas DEP-1 in accordance with its suggested role as tumor suppressor (59, 60) interacts with members of the catenin family, PTPβ does not recognize these substrates (61). In a recent study, PTPβ was shown to dephosphorylate the angiopoietin receptor Tie-2 (62). At present it is unclear whether this indicates that PTPβ is also a tumor suppressor or if specific inhibitors of this PTP potentially could play a role in the clinic. It should, however, be emphasized that PTPβ was not selected for these studies with the aim of developing a novel drug candidate as such, but due to the fact that it differs phylogenetically and structurally from PTP1B. We speculate that if successful in designing inhibitors that are selective for a PTP that is only distantly related to PTP1B, the approach is general in nature, thus potentially allowing design of selective inhibitors against most known mammalian PTPs.

We have identified previously (29) several selectivity determining regions in PTPs. In particular, the region defined by residues 47, 48, 258, and 259 seems attractive from a structure-based design viewpoint. This region is also critical for correct positioning of substrates in PTP1B (63). The interest for this area of PTP1B was in particular spurred by (i) the finding of a second aryl phosphate-binding site (64), and (ii) the observation that two tandem Tyr(P) residues in the insulin receptor activation loop simultaneously bind to this site and the active site (65). To address both sites, the insulin receptor substrate has to pass through a cleft-like part of PTP1B, the 258–259 gateway (66). We have used this gateway in design of highly selective PTP1B inhibitors that fit in PTP1B and the highly homologous T cell-PTP, but not in PTPs with bulky residues in position 259 (30).

Residue 48 has also received much attention. Thus, Barford and co-workers (67) in an early study provided the framework for rational design of inhibitors that bind to residues 47 and 48. In our laboratory, we have designed highly selective PTP1B inhibitors that form a salt bridge with the negatively charged carboxyl group of Asp-48 in PTP1B (19). Recently, the Abbott PTP group, also using an oxamic acid-based compound as synthetic starting point, have designed highly potent inhibitors that address Asp-48 in PTP1B, the 258–259 gateway, and the above 2nd aryl phosphate-binding site (15, 21). In this context, it is of interest that an elegant study demonstrated that Arg-47 can adopt one of two conformations and thus accommodate different substrates that bind to this part of PTP1B. Indeed, several PTP1B inhibitors have been shown to address Arg-47 (18, 22).

Although the above approaches utilizing the 258–259 gateway and the 2nd aryl phosphate-binding site are excellent for design of highly selective and potent PTP1B inhibitors, these regions seem less useful for design of compounds that selectively inhibit other PTPs. Based on our previous experience with OATP as synthetic scaffold (30), we reasoned that this compound could also be used as synthetic starting point for the design of selective inhibitors of PTPβ. However, in this case molecular modeling indicated that position 5 in OATP would be more attractive for introducing substituents that could address the region defined by residues 47 and 48. Of note, a highly selective and potent PTP1B inhibitor that simultaneous binds to the active site and both residues was recently described (22). Therefore, our goal was to introduce substituents into OATP that would bind PTPβ with high affinity and PTP1B with lower affinity. A number of substituents were tested, and phthalimide-based side chains seemed particularly useful.

In contrast to our predictions, one compound that contained an OH group in position 4 of the phthalimide side chain was equipotent against PTPβ and PTP1B. Combined use of x-ray
crystallography, molecular modeling, and reciprocal mutational analyses revealed that this compound attained completely different conformation in the two enzymes, and most importantly that the OH group was essential for PTPβ1 binding but dispensable for binding to PTPβ. Therefore, by removing or repositioning of the OH group, we have been able to synthesize inhibitors that are highly selective for PTPβ over PTPβ1. Most important, when tested against a set of other PTPs, substantial selectivity is still observed. We believe this is the first example of structure-based design of selective inhibitors of other PTPs than PTPβ1.

In conclusion, we have succeeded in generating highly potent, low molecular weight, and non-phosphorous inhibitors of PTPβ, and simultaneously we introduced a very high degree of selectivity against PTPβ1 and several other PTPs representing a broad spectrum of this class of enzymes. Additionally, this study reveals the prospect for the design and development of selective and low molecular weight PTP inhibitors from a general inhibitor scaffold. Our findings provide support for the notion that highly selective and potent inhibitors can be developed for a broad range of mammalian PTPs.

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