Suramin Derivatives as Inhibitors and Activators of Protein-tyrosine Phosphatases*

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Protein-tyrosine phosphatases (PTPs) are important signaling enzymes that have emerged within the last decade as a new class of drug targets. It has previously been shown that suramin is a potent, reversible, and competitive inhibitor of PTP1B and Yersinia PTP (YopH). We therefore screened 45 suramin analogs against a panel of seven PTPs, including PTP1B, YopH, CD45, Cdc25A, VHR, PTPα, and LAR, to identify compounds with improved potency and specificity. Of the 45 compounds, we found 11 to have inhibitory potency comparable or significantly improved relative to suramin. We also found suramin to be a potent inhibitor (IC50 = 1.5 μM) of Cdc25A, a phosphatase that mediates cell cycle progression and a potential target for cancer therapy. In addition we also found three other compounds, NF201, NF336, and NF339, to be potent (IC50 < 5 μM) and specific (at least 20–30-fold specificity with respect to the other human PTPs tested) inhibitors of Cdc25A. Significantly, we found two potent and specific inhibitors, NF250 and NF290, for YopH, the phosphatase that is an essential virulence factor for bubonic plague. Two of the compounds tested, NF504 and NF506, had significantly improved potency as PTP inhibitors for all phosphatases tested except for LAR and PTPα. Surprisingly, we found that a significant number of these compounds activated the receptor-like phosphatases, PTPα and LAR. In further characterizing this activation phenomenon, we reveal a novel role for the membrane-distal cytoplasmatic PTP domain (D2) of PTPα: the direct intramolecular regulation of the activity of the membrane-proximal cytoplasmatic PTP domain (D1). Binding of certain of these compounds to PTPα disrupts D1-D2 basal state contacts and allows new contacts to occur between D1 and D2, which activates D1 by as much as 12–14-fold when these contacts are optimized.

Suramin, one of the oldest synthetic therapeutics, has long been used for the treatment of sleeping sickness and onchocerciasis (1). Within the last decade, suramin has shown several other potentially therapeutic properties such as the inhibition of HIV infection, the inhibition of growth factor/receptor interactions, and the inhibition of angiogenesis. In addition this laboratory has previously shown that suramin is a potent inhibitor of protein-tyrosine phosphatases (PTPs) (2), which is consistent with the observation that suramin leads to enhanced levels of tyrosine phosphorylation in several cell lines (3, 4). PTPs function to remove the phosphoryl group from tyrosine-phosphorylated proteins. Originally it was suspected that PTPs were simply relatively nonspecific housekeeping enzymes that existed to turn off the pathways that were turned on by the tyrosine kinases. It is now known that of the ~100 PTPs encoded in the human genome, many have very specific regulatory functions in all aspects of cellular function, such as cell growth, metabolism, and cell division. In fact, several PTPs have emerged as attractive drug targets (5). Substantial evidence indicates that inhibitors of PTP1B, which dephosphorylates and inactivates the insulin receptor, could be used to treat diabetes and obesity (6, 7). Inhibitors of YopH, the PTP that is an essential virulence factor of *Yersinia pestis*, could be used in the treatment of bubonic plague (8). Inhibitors of CD45, which has been found to play a role in cancer and in the immune response, may potentially be used as cancer chemotherapy and diseases of autoimmunity (9). Inhibitors of Cdc25, a dual specificity phosphatase that mediates cell cycle progression, could potentially serve as a cancer or perhaps even Alzheimer’s treatment (10, 11).

Despite their attractiveness as drug targets, relatively few potent and specific inhibitors of PTPs have been reported. In fact, at one time it was widely held that finding potent and specific inhibitors of PTPs would be impossible because of the highly conserved nature of the PTP active site. Although some strides have been made within the last few years in overcoming this obstacle, the search for specific inhibitors remains a major challenge of the field. Currently there are only a few reported highly potent and specific PTP1B (12) and YopH (13) inhibitors. No potent or specific inhibitors are described for PTPα, LAR, or CD45, and until recently, the most potent Cdc25 inhibitors were in the low micromolar range, often with low or undetermined specificity (14–23).

Because suramin is already in clinical use, and it inhibits PTPs, it is potentially an attractive treatment of diseases mediated by PTPs, such as diabetes, cancer, and bubonic plague. However, its demonstrated effects on many diverse pathways would certainly lead to unwanted side effects. We reasoned that slight variations in the suramin structure may lead to PTP inhibitors with improved potency and specificity without sac-
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rificing its desirable absorption and distribution properties. Therefore, we obtained a library of suramin-like compounds and screened them for inhibitory activity against a panel of clinically important phosphatases, PTP1B, YopH, CD45, LAR, PTPs, VHR, and Cdc25A. We report several compounds with markedly improved potency and specificity over suramin in inhibiting PTP1B and CD45, several potent and specific inhibitors of Cdc25A and YopH, and interestingly, several compounds that activate the PTP activity of LAR and PTPs. In doing so, we provide novel lead structures for further optimization to generate tools to study the in vitro PTP function and additional evidence that it is possible to obtain potent and specific PTP inhibitors despite the fact that these enzymes all share the same active site architecture.

In further characterizing the activation of PTPs by these compounds, we reveal a novel role for the membrane-distal cytoplasmic domain (D2) of PTPs: the intramolecular regulation of the activity of the membrane-proximal domain (D1). We show that these compounds do not affect the activity of PTPs-D1 expressed alone and that mutations aimed to disrupt D1-D2 interactions in the linker/spacer regions cause enhanced affinity for and activation by these compounds. This is a particularly significant finding in that most studies of receptor-like PTPs suggest that the major role for the D2 domain is in mediating dimerization-induced inhibition. Any activating functions of the D2 domain have been attributed to the blocking of this dimerization-induced inhibition (24, 25). Our results do not contradict these findings but rather show that D2 can play an additional role in the direct intramolecular regulation of D1 activity through a conformational change.

**EXPERIMENTAL PROCEDURES**

**Suramin and Its Analogs**—Suramin (sodium salt) was obtained from Sigma. The suramin analogs employed in this study were synthesized by methods described previously (26–28). Structures were confirmed by 1H NMR, 13C NMR, and MS (fast atom bombardment) (29). The purity of the compounds was confirmed by TLC and HPLC (30). Some of the compounds were synthesized years ago. Repeated HPLC investigations showed the stability of the compounds after a long storage at room temperature. Even in aqueous solution (pH 7.4), degradation appears to proceed very slowly (21).

**Protein Expression and Purification**—PTP1B (32), Cdc25A (33, 34), LAR, CD45 (12, 35), PTPs, PTP-D1, PTP-D2 (36), and VHR (37) were expressed in Escherichia coli BL21 (DE3) cells and purified according to the previously published procedures at 4 °C. Mutant PTPs enzymes (416AA, 4946V, and 529AA) were constructed according to the QuikChange mutagenesis protocol (Stratagene). The mutants were purified as the wild type enzyme. The PTPs enzymes and mutants used for the limited proteolysis and activation assays were subjected to the additional step of ion exchange chromatography using a 1-ml MonoQ column on an AKTA purifier fast protein liquid chromatography (Amersham Biosciences) in pH 8.5 Tris buffer.

**Enzyme Inhibition Assays**—All of the assays were done at 25 °C and pH 7.0 using p-nitrophenyl phosphate (pNPP) (Sigma) as the substrate in 100-µl reaction volumes in 1.1-m1 polypropylene tubes (Marsh Biomedical). The buffer used was 50 mM 3,3-dimethyl glutarate with the ionic strength adjusted to 150 mM with sodium chloride. The assays for the limited proteolysis and activation assays were subjected to the QuikChange mutagenesis protocol (Stratagene). The mutants were purified as the wild type enzyme. The PTP enzyme and screened them for inhibitory activity against a panel of clinically important phosphatases, PTP1B, YopH, CD45, LAR, PTPs, VHR, and Cdc25A. We report several compounds with markedly improved potency and specificity over suramin in inhibiting PTP1B and CD45, several potent and specific inhibitors of Cdc25A and YopH, and interestingly, several compounds that activate the PTP activity of LAR and PTPs. In doing so, we provide novel lead structures for further optimization to generate tools to study the in vitro PTP function and additional evidence that it is possible to obtain potent and specific PTP inhibitors despite the fact that these enzymes all share the same active site architecture.

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**RESULTS AND DISCUSSION**

**Compounds with the Suramin Core Structure**—Fig. 1 shows the structure of suramin along with a table that lists the variants of suramin that were employed in this study. Also shown are the IC50 values obtained with PTP1B, Yersinia PTP (YopH), CD45, Cdc25A, and VHR. The measured IC50 value of 11 µM for suramin against PTP1B agrees well with the Kd value of 4 µM obtained previously (2). When an inhibitor binds reversibly and competitively, as suramin does to PTP1B, the IC50 value determined when the substrate concentration is equal to the Kd is twice the IC50 value as shown under “Experimental Procedures.” In this study, the substrate (pNPP) concentration employed for each PTP was equal to the Kd of the enzyme. We tested several of the suramin analogs against several of the PTPs used in this study. In all cases the inhibition was found to be reversible and competitive. Additionally, none of the suramin analogs have any functionality that would be expected to irreversibly inactivate PTPs. Therefore, all of the IC50 values reported in this study should directly reflect the affinity of the compound for the PTPs tested. Surprisingly none of the compounds shown in Fig. 1 nor any of the compounds employed in this study were found to inhibit LAR or PTPs, but rather many were found to moderately activate these phosphatases. Thus, for LAR and PTPs, the relative phosphatase activity at 10 µM compound concentration is shown. This series of compounds was used in an attempt to gain a structure activity relationship of the suramin analogs to determine which parts of suramin were required for inhibitory activity and which parts could tolerate major variations.

For the most part, all variations of the suramin core structure tested here were well tolerated. For PTP1B, similar IC50 values were obtained with suramin and the other compounds with suramin core structures. Substitutions at the R2 position had little effect on the affinity of the compounds for the phosphatases tested. Interestingly, even changing the naphthyl trisulfonate moiety into phenyl sulfonate (NF062) did not ap-
preciably change the IC_{50} value for PTP1B. In general, the same trend was observed for the other PTPs examined in this study except for CD45, which showed a 5-fold increase in the IC_{50} values for NF062. It was suspected previously that the high affinity of suramin for PTPs was not simply due to the presence of an aryl sulfonate moiety, which would potentially serve as a phosphotyrosine mimic and bind to the active site of PTPs (2). Because the \(K_i\) value of sulfosalicylic acid for \textit{Yersinia} PTP was 5,700-fold larger than that of suramin, it was concluded that other structural features of suramin, such as the additional aryl sulfonate ring of the naphthyl sulfonate moiety or the suramin core structure, were important for high affinity binding. Our results suggest that it is the suramin core structure rather than the additional aryl sulfonate ring that is important for high affinity PTP binding because NF062 retains the ability to bind with high affinity to PTPs, although it is missing this additional ring.

It also appears that both halves of the symmetrical suramin core structure are required for high affinity binding to all PTPs studied. The compound NF520 (Fig. 1), which is one-half of the suramin structure minus the central urea group, did not significantly inhibit any of the PTPs used in this study. This requirement for both halves of the molecule for activity has been noted in other systems involving suramin, such as the inhibition of angiogenesis by suramin and its analogs (38).

Interestingly, suramin was found to bind with high affinity to the dual specificity phosphatase Cdc25A, but not VHR, another dual specificity phosphatase. The IC_{50} value of suramin (1.5 \(\mu\)M) for Cdc25A makes it as good or better than most of the Cdc25 inhibitors reported to date (14–23). Cdc25A along with

| Compound     | \(R_1\)       | \(R_2\)     | IC_{50} (\muM) | Relative activity at 10 \(\mu\)M compound |
|--------------|---------------|-------------|----------------|------------------------------------------|
|              |               |             | PTP1B | YopH | CD45 | Cdc25A | VHR | PTPα | LAR |
| Suramin      | naphthyl-trisulfonate | methyl       | 11±1 | 7±1  | 8.5±0.7 | 1.5±0.2 | >100 | 1.32 | 1.26 |
| NF062        | phenyl sulfonate | methyl       | 10±4 | 12±3 | 43±5   | 2.8±0.7 | >100 | 1.12 | 1.11 |
| NF127        | naphthyl-trisulfonate | ethyl       | 9±1  | 11±3 | 7.8±0.5 | 2.6±0.6 | >100 | 1.34 | 1.3  |
| NF145        | naphthyl-trisulfonate | t-butyl     | 7±1  | 13±3 | 14±1   | 4.9±0.9 | >100 | 1.32 | 1.32 |
| NF151        | naphthyl-trisulfonate | isopropyl   | 9±2  | 13±4 | 13±2   | 5±1     | >100 | 1.31 | 1.34 |
| NF520        | -             | -           | >100 | >100 | no inh. | >100     | >100 | 0.95 | 1.05 |

Fig. 1. Compounds with suramin core structure.
Cdc25B and Cdc25C are dual specificity phosphatases that dephosphorylate and activate cyclin-dependent kinases, thereby driving progression through the cell cycle (39–41). Cdc25 enzymes have been shown to be potential oncogenes under certain circumstances (42). Cdc25A and Cdc25B have also been shown to be overexpressed in several types of cancer cells (43–50). Importantly, it has also been shown that treatment of tumor cell lines with Cdc25 inhibitors inhibits tumor growth or causes cell cycle arrest (15, 16, 51–53), which validates Cdc25 as a drug target and makes inhibitors of Cdc25 very valuable. It has been known for some time that suramin treatment increases the tyrosine phosphorylation levels of Cdc2, a cyclin-dependent kinase responsible for initiation of mitosis (54). Because the function of Cdc25 is to remove the inhibitory phosphorylations in Cdc2 on tyrosine 15 (as well as threonine 14), it is tempting to speculate that suramin leads to enhanced tyrosine phosphorylation of Cdc2 by inhibiting Cdc25. Further, because it has been shown that inhibiting Cdc25 leads to cell cycle arrest, it is possible that the antitumor activity of suramin may be due in part to the inhibition of Cdc25.

**Slight Variations of the Suramin Core Structure**—The compounds NF171 and NF280 (Fig. 2) are virtually identical to suramin except that the intermediate rings contain para-linkages rather than meta-linkages as in suramin. NF279 has para-linkages in the innermost rings as well as in the intermediate rings. Interestingly, as the bottom panel of Fig. 2 indicates, these slight variations can have substantial effects on the inhibitory properties of these molecules. NF171 differs from suramin only in that the terminating naphthyl trisulfonate

| Compound | IC$_{50}$ (µM) | Relative activity at 10 µM compound |
|----------|----------------|----------------------------------|
|          | PTP1B | YopH | CD45 | Cdc25A | VHR | PTPα | LAR |
| NF171    | 3.7±0.8 | 7±3  | 17±2 | 1.8±0.4 | >100 | 1.32 | 1.29 |
| NF279    | 2.1±0.5 | 2.8±0.8 | 1.7±0.2 | 1.0±0.3 | >100 | 1.12 | 1.11 |
| NF280    | 1.6±0.3 | 2.0±0.2 | 3.8±0.8 | 0.84±0.28 | >100 | 1.34 | 1.3  |

Fig. 2. Compounds with slight variations of the suramin core structure.
moiety is connected to the para-position of the intermediate ring, yet it shows roughly a 3-fold enhancement and 2-fold reduction in binding affinity for PTP1B and CD45, respectively. NF280 is the same as NF171 except that it is missing the methyl groups on the intermediate rings, which leads to a 7-fold enhancement in affinity for PTP1B, a 3-fold enhancement for YopH, and 2-fold enhancements in binding affinity for CD45 and Cdc25A relative to suramin. NF279, which is identical to NF280 except that it has para-linkages in its innermost rings as well as in its intermediate rings, shows similar inhibition properties as NF280.

**Suramin Analogs with Different Bridging Functionalities**—NF059 and NF066 (Fig. 3) are identical to suramin except that the bridging urea group has been replaced by fumaric acid diamide and isophthalic acid diamide groups, respectively. NF066 shows only a modest 2-fold improvement in its affinity for PTP1B, but no significant improvements for any other PTPs. On the other hand, NF059 shows a 4-fold enhancement in its affinity for PTP1B, YopH, and Cdc25A. The IC₅₀ of 0.38 μM makes NF059 one of the best Cdc25A inhibitors reported to date. Further, NF059 shows significant selectivity for Cdc25A, with 120-, 13-, 6-, and 4-fold selectivity over VHR, CD45, PTP1B, and YopH, respectively.

**Different Core Structures with Enhanced Binding**—NF506 is identical to suramin except that the intermediate rings are benzimidazole groups (Fig. 4). NF504 is identical to NF506 except that the naphthyl trisulfonate groups are replaced by phenyl disulfonate groups. As the IC₅₀ values for NF506 indicate, the inclusion of this benzimidazole ring markedly enhances the inhibitory properties of the molecule for all PTPs (except LAR and PTPᵣ) leading to 48-, 70-, 10-, 2-, and ≥3-fold enhancements in affinity with respect to suramin for PTP1B, YopH, CD45, Cdc25A, and VHR, respectively. The IC₅₀ of 90 nM for YopH makes NF506 one of the most potent YopH inhibitors reported. Further, it shows a moderate degree of specificity: 3-fold over PTP1B, 4-fold over CD45, 9-fold over Cdc25A, and ≥300-fold over VHR. Changing the terminating ring to phenyl disulfonate as in NF504 from naphthyl trisulfonate as in

![NF059](image1.png)

![NF066](image2.png)

| Compound | IC₅₀ (μM) | Relative activity at 10 μM compound |
|----------|----------|-----------------------------------|
|          | PTP1B    | YopH    | CD45    | Cdc25A  | VHR    | PTPᵣ    | LAR     |
| NF059    | 2.3±0.8  | 1.5±0.3 | 4.9±0.5 | 0.38±0.04 | 48±6   | 1.43    | 1.39    |
| NF066    | 5±1      | 8.0±0.7 | 4.9±0.7 | 2.7±0.6 | >100   | 1.01    | 1.24    |

**Fig. 3.** Suramin-like compounds with different central bridging groups.
NF506 leads to a 8-fold decrease in affinity for YopH and a modest 2-fold decrease in affinity for PTP1B, CD45, and VHR and leaves the affinity unchanged for Cdc25A. Thus, as in the results shown in Fig. 1, it is the core structure rather than the terminating ring that is most important for high affinity binding.

A Phenyl Sulfonate-containing Molecule with Enhanced Binding—NF110 (Fig. 5) differs significantly from suramin, and the molecules mentioned previously in that it has four phenyl sulfonate branches that emanate from the central core. This molecule shows 2-, 9-, and 3-fold improvements in the IC₅₀ values for PTP1B, CD45, and Cdc25A, respectively, over suramin. This is the only molecule that we found that deviates significantly from the suramin-like structure composed of a bridging group, inner rings, intermediate rings, and outer rings that shows improved potency. The bridging urea group along with two of the outer rings form a structure similar to the suramin core structure. However, it appears that all parts of this molecule are important for high affinity binding, because molecules like NF250 and NF290 (Fig. 6), which contain the bridging group and two outer rings, show little inhibition.

PTP1B-, YopH-, and Cdc25A-specific Inhibitors—Although the inhibitors mentioned so far show markedly improved potency over suramin in inhibiting PTPs, they are all relatively

| Compound | IC₅₀ (µM) | Relative activity at 10 µM compound |
|----------|----------|-----------------------------------|
|          | PTP1B    | YopH | CD45 | Cdc25A | VHR | PTPα | LAR |
| NF504    | 0.49±0.05 | 0.7±0.1 | 0.7±0.1 | 0.56±0.15 | 80±17 | 1.4 | 1.54 |
| NF506    | 0.25±0.05 | 0.09±0.02 | 0.35±0.07 | 0.79±0.25 | 30±6 | 1.65 | 1.64 |

Fig. 4. Compounds with core structures that show enhanced binding.
nonspecific, save for NF059 for Cdc25A and NF506 for YopH, which showed significant specificity. We did, however, discover some additional molecules that were specific for certain PTPs: one for PTP1B, one for YopH, and three for Cdc25A. NF069 (Fig. 6) inhibits PTP1B with an IC50 of 9.6 μM, which is comparable in potency with suramin. It is significantly more specific than suramin, however, showing at least 10-fold specificity over YopH, CD45, Cdc25A, PTPα, and LAR and 7-fold specificity over VHR. This compound is significantly different than any of the other inhibitors in that it is a phenyl phosphonate-containing compound. It shows some resemblance to the other PTP1B-specific phosphonate-containing inhibitors reported in other studies (55). Previously, a crystal structure of PTP1B in complex with aromatic substrates revealed two aryl phosphate-binding pockets: one at the active site and one at the second site adjacent to the active site (32). It is possible that NF069 interacts simultaneously with the active site and an adjacent nonconserved peripheral site in PTP1B and that this is the basis for the specificity of this inhibitor. However, the moderate potency would indicate that these interactions are not optimal.

NF290 (Fig. 6) was found to be a potent and specific inhibitor of YopH. It had an IC50 value of 4.2 μM, with at least 16- and 12-fold selectivity over PTP1B and Cdc25A and at least 24-fold selectivity over CD45, VHR, PTPα, and LAR. In addition, as shown in Fig. 4, NF506 is one of the most potent YopH inhibitors (IC50 = 90 nM) identified to date (13). These results are important given the increasing risk of misuse of infectious agents, such as Y. pestis, as weapons of terror, as well as instruments of warfare for mass destruction (56–58). Thus, there is an urgent need to devise effective protective strategies that could be implemented soon after a bioterrorist attack. YopH has been identified as an essential Yersinia virulence factor that inhibits macrophage phagocytosis (59). This is consistent with the observation that YopH dephosphorylates several components of the focal adhesion complex (e.g. p130Cas, FAK, and paxillin), compromising the cellular infrastructure necessary to mediate phagocytosis (60, 61). Because the tyrosine phosphatase activity of YopH is essential for the virulence of Yersinia (8, 62), specific inhibitors targeted to the YopH Yersinia PTP are expected to render the bacteria avirulent as shown by Liang et al. (13). Thus, potent and specific inhibitors for the Yersinia PTP should serve as effective agents to block the spread and proliferation of the Y. pestis infection.

Three potent and specific Cdc25A inhibitors were found in this study: NF201, NF336, and NF339 (Fig. 6). NF201 inhibits Cdc25A with an IC50 of 2.0 μM and at least 50-fold specificity over CD45, VHR, PTPα, and LAR and 35-fold specificity over PTP1B. Similarly, NF336 has an IC50 of 3.2 μM for Cdc25A and shows at least 30-fold specificity for Cdc25A over all of the PTPs tested in this study, whereas NF339 has an IC50 of 4.2 μM with 20-fold specificity over PTP1B and at least 25-fold specificity over the other PTPs. These findings are significant because few potent and specific Cdc25 inhibitors have been reported to date, and such inhibitors are potentially useful in studying the cell cycle or treating diseases such as cancer. Although it is unclear whether these suramin derivatives are cell permeable, suramin itself has been used clinically for the treatment of trypanosomiasis and onchocerciasis since the 1920s. Limited cellular uptake of suramin has been proposed to occur by the process of receptor-mediated fluid phase endocytosis (63). Clearly, suramin derivatives showing selectivity for

| Compound | IC50 (μM) | Relative activity at 10 μM compound |
|----------|-----------|-----------------------------------|
|          | PTP1B    | YopH    | CD45    | Cdc25A  | VHR    | PTPα   | LAR    |
| NF110    | 4.5±1     | 5±2     | 0.9±0.2 | 0.46±0.10 | >100   | 1.02   | 1.18   |
| NF250    | No inh.   | 30±10   | No inh. | >100    | >100   | 1.04   | 1.06   |

NF110, a four-branched molecule with enhanced inhibitory properties.
PTP1B, YopH, and Cdc25A will serve as excellent starting points for future development of small molecule inhibitory agents that possess the desirable potency, selectivity, and cell permeability required for pharmacological interrogation of these PTPs in vivo.

**Activation of Two Receptor-like PTPs by Suramin and Several of Its Analogs**—Interestingly, no inhibitors of PTPα or LAR were found in this study. Instead, many of the compounds that inhibit PTP1B, YopH, CD45, and Cdc25A were found to activate PTPα and LAR (Figs. 1–6). For suramin itself the activa-

| Compund   | IC₅₀ (µM) | Relative activity at 10 µM compound |
|-----------|-----------|-----------------------------------|
|           | PTP1B     | YopH    | CD45   | Cdc25A | VHR | PTPα | LAR |
| NF069     | 9.6±3     | >100    | >100   | >100   | 74±40 | 1.01 | 0.99 |
| NF201     | 70±10     | 20±10   | 100±30 | 2.0±0.2 | >100 | 0.98 | 1.09 |
| NF290     | 70±10     | 4.2±0.3 | no inh.| 49±6   | >100 | 0.99 | 0.98 |
| NF336     | >100      | >100    | no inh.| 3.2±0.3 | >100 | 0.99 | 0.99 |
| NF339     | 80±10     | 45±15   | >100   | 4.2±0.8 | >100 | 0.99 | 0.98 |

**Fig. 6.** Cdc25A-, YopH-, and PTP1B-specific inhibitors.
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**Table I** Kinetic parameters of PTPα and its mutants with pNPP as a substrate

| PTPα     | $k_{cat}$ s$^{-1}$ | $K_m$ for pNPP μM | $K_m$ for NF506 μM | Maximal activation by NF506 fold |
|----------|-------------------|-----------------|----------------|-------------------------|
| Wild type| 1.26 ± 0.07       | 2.9 ± 0.7       | 32 ± 7         | 4.5 ± 0.3              |
| 416AA    | 0.77 ± 0.05       | 2.8 ± 0.7       | 25 ± 10        | 2.0 ± 0.2              |
| 529AA    | 1.25 ± 0.05       | 2.9 ± 0.5       | 64 ± 7         | 11.6 ± 0.8             |
| G496V    | 1.74 ± 0.09       | 4.6 ± 1.4       | 5.2 ± 0.6      | 13.5 ± 0.3             |

**Fig. 7.** Activation curves for PTPα (circles) and its mutants, 416AA (squares), 529AA (triangles), and G496V (diamonds) in response to NF506.

...tion was rather moderate: only about 30% at 10 μM suramin. NF506 (Fig. 4) was found to be the most potent activator of PTPα, showing over 1.6-fold activation at 10 μM. Further, this activation was saturable, giving a $K_d = 33 \mu M$ and a maximal activation of 4.5-fold when the data were fit to a binding curve as described under “Experimental Procedures” (Table I and Fig. 7). Both LAR and PTPα are receptor-like PTPs that contain two cytosolic PTP domains called D1 and D2. For the receptor-like PTPs, the membrane-proximal domain (D1) contains most of the phosphatase activity, but in PTPα the membrane-distal domain (D2) also contains significant (10% of D1) phosphatase activity (36, 64, 65). Interestingly, this activation phenomenon is not common to all receptor-like PTPs, because CD45 is also a receptor-like PTP, yet it was inhibited by these compounds.

**Both D1 and D2 Are Required for PTPα Activation**—Because receptor-like PTPs contain two cytosolic PTP domains, we wanted to determine whether both of these domains were required for this activation phenomenon. In this study, the receptor like PTPs employed contain the cytosolic D1 and D2 in tandem minus the extracellular and transmembrane domains. The term PTPα refers to the PTPα D1 and D2 domains (residues 167–793) expressed in tandem (as shown in Fig. 8B). Because both of the PTP domains in PTPα display phosphatase activity, we also expressed and purified PTPα-D1 (residues 167–503) and PTPα-D2 (residues 504–793) separately (65) and screened the compounds against these phosphatases for inhibitors and activators. We found neither substantial activators nor inhibitors of either PTPα-D1 or PTPα-D2. This indicates that both domains are required for PTPα activation by these compounds.

**Conformational Activation of PTPα**—We suspected that binding of the compounds to PTPα causes a conformational change that activates the phosphatase activity of PTPα that can only occur when both domains are present. To test whether or not a conformational change occurs upon binding to NF506, we performed trypsin cleavage and SDS-PAGE and used in-gel digestion and liquid chromatography-MS/MS peptide sequencing to identify regions in PTPα that show enhanced protease sensitivity in the presence of NF506. Fig. 8A shows the SDS-PAGE analysis of such a digestion. *Lane 1* shows molecular mass standards; *lanes 2–7* are 5 μM PTPα incubated with 0.05 ng/μl trypsin for 0, 5, 10, 15, 30, and 45 min, respectively; and *lanes 8–13* are identical to *lanes 2–7*, respectively, except that 100 μM of NF506 was also included. Judging by the intensity of the highest molecular mass band (the intact PTPα) in each lane, NF506 causes an overall increase in trypsin sensitivity. After 45 min in the presence of 0.05 ng/μl trypsin with no NF506 (*lane 7*), it appears that at least 50% of the PTPα remains intact. However, after 45 min in the presence of 0.05 ng/μl trypsin with 100 μM NF506 (*lane 13*), it appears that less than 25% of the intact protein remains. Clearly binding of NF506 to PTPα leads to an overall increase in protease sensitivity, most likely as a result of a conformational change. We therefore conclude that NF506 binds to PTPα and induces a more active conformation.

A closer inspection of Fig. 8A reveals that several of the bands corresponding to partially digested PTPα are much more prominent in the presence of 100 μM NF506. We cut several of these bands out and subjected them to in-gel trypsin digestion and liquid chromatography-MS/MS peptide sequencing following standard published procedures. It is important to keep in mind that in these experiments, it is rare to obtain 100% sequence coverage of the band of interest, so it is not possible to unambiguously determine the exact cleavage site. Therefore, the purpose of these experiments was to narrow down the general region or regions of PTPα that become more susceptible to cleavage and use this information along with the crystal structures of receptor-like PTPs to guide our site-directed mutagenesis studies. In all we focused on six bands that showed increased prominence in the presence of NF506 (labeled as *bands 1–6* in Fig. 8A). We found that bands 1 and 2 encompassed an N-terminal portion of PTPα corresponding to residues 188–530 and 183–530, respectively, suggesting that these bands resulted from proteolytic cleavage at residue 530 or after. Band 3 encompassed residues 188–565, suggesting that cleavage at residue 565 or after generated this band. Bands 4–6 all encompassed residues 440–756, suggesting that cleavage at or before residue 440 generated these bands. Fig. 8B shows the potential cleavage sites (represented as *arrows*) that generated bands 1–6 mapped onto a schematic of PTPα. Interestingly, these potential cleavage sites, residues 440, 530, and 565, are all at or close in primary structure to the so-called spacer region of PTPα, which lies between D1 and D2. Because all of these bands were chosen based on their increased prominence in the presence of NF506, we conclude that NF506 causes increased proteolytic susceptibility and flexibility in this region (i.e. the C-terminal portion of D1, the spacer region, and the N-terminal portion of D2).

**Mutational Analysis of PTPα Activation**—Given the fact that binding of NF506 to PTPα causes the region between D1 and
D2 to be more proteolytically labile and that both D1 and D2 domains are required for this activation phenomenon, we attempted to introduce mutations into PTPα/H9251 that could perturb the intramolecular interactions between D1 and D2 and therefore the activation properties of this enzyme. We employed the results of our proteolysis experiments and the crystal structure of LAR (66) to guide our mutagenesis. LAR is the only receptor-like PTP for which a crystal structure that includes both D1 and D2 in tandem is available. Furthermore, LAR has high sequence and structural homology to PTP/H9251 (66–68), and we have shown that it is activated by the same compounds that activate PTP/H9251. Therefore, the crystal structure data of LAR is highly relevant for generating mutants in PTP/H9251. The crystal structure of LAR reveals extensive interactions between D1 and D2, in which 650 Å² surface is buried at the interface. Additionally, it was found that the linker region, residues Gly1585–Glu1588 (Gly496–Glu499 in PTP/H9251), makes a sharp turn that appears to lock the relative orientations of the two domains in place. It was postulated that a glycine residue is necessary at this position because it is the only amino acid that can adopt the necessary conformation to make this turn. Importantly, all of the residues involved in these interdomain contacts are conserved in most receptor-like PTPs (66).

We chose to make and characterize the following mutants of PTPα/H9251:

- K416A/K417A (referred to as 416AA), G496V, and K529A/K530A (referred to as 529AA). Lysines 416 and 417 correspond to arginines 1505 and 1506 in LAR, which are located in D1 and are involved in a salt bridge and extensive van der Waals' contacts with D2. Mutation of these two residues to alanine should destabilize the D1-D2 interface. Glycine 496 corresponds to glycine 1585 in LAR, the glycine that is critical for the turn made by the linker region. Replacement of this glycine with a bulky, β-branched valine residue should greatly destabilize if not preclude this turn altogether. Finally, we chose to mutate lysines 529 and 530 into alanines based upon our limited proteolysis results, which suggest that these residues are potential trypsin cleavage sites that become even more susceptible to cleavage in the presence of NF506.

The kinetic and binding parameters of PTPα and its mutants are summarized in Table I, and the binding curves in response to NF506 are shown in Fig. 7. Importantly, all three mutants displayed $k_{cat}$ and $K_m$ values that were comparable with wild type PTPα, indicating that none of these mutations significantly perturbed the active site structure of the enzyme or the basal state activity of the enzyme. These mutants did, however, show significant differences from the wild type enzyme in their response to NF506. The wild type PTPα bound NF506 with a $K_a$ of 33 μM and a maximal activation of 4.5-fold. The 416AA mutant had a $K_a$ that was reduced 1.3-fold as compared with wild type; however, the maximal activation by NF506 was also...
The effects of the mutations examined in this study can be understood in terms of thermodynamics. Mutations such as G496V and 529AA that may destabilize this D1-D2 interface would lead to a proportionate shift in the equilibrium toward the activated state (the top right-hand corner of Fig. 9). Overall the activated state is still highly unfavored, however, even in the case of these mutants because these mutants do not show significantly enhanced kinetic parameters (relative to wild type PTPα) in the absence of NF506. The effects of the mutations only manifest themselves in the activation parameters in response to NF506. Perhaps the mutants are able to destabilize the D1-D2 contacts in the basal state but are insufficient to promote the new D1-D2 contacts in the activated state. We can rationalize the lack of activation in the 416AA mutant because these residues exist on an α-helix that is connected to the active site on the D1 domain. Perhaps in the activated state of PTPα, lysines 416 and 417 are required for maintaining the proper conformation of the active site.

**Significance of PTPα Activation**—The finding that PTPs and some of its mutants can be activated up to 12–14-fold is significant for several reasons. Most receptor-like PTPs contain two intracellular PTP domains: D1 and D2. The phosphatase activity resides primarily in the membrane-proximal D1 domain. Despite the fact that the membrane-distal D2 domain does not appear to be catalytically active, there is a growing body of evidence that D2 may perform important functions in *vivo*. One of the functions is to serve as a docking site for downstream signaling proteins (71, 72). Another function of D2 appears to be regulation of the PTP activity of D1. For example, D2 domains of several receptor-like PTPs were found to bind to and inhibit the membrane-proximal D1 domains of PTPs and PTPσ, a LAR-related PTP (73, 74). The D2 domain of PTPδ was shown to directly inhibit PTPδ-D1 activity concomitant with binding to the juxtamembrane sequence of PTPs (74). It was recently shown that approximately one-fourth to one-third of the cytosolic form of PTPs exists as an inactive dimer in *vitro*, and that oxidative stress enhances this dimerization and inhibition, both in *vitro* and in *vivo*. Additionally it was shown that this dimerization and inhibition is largely mediated through intermolecular D2-D2 interactions (75). Similar redox regulation of D2-D2 dimerization-mediated inhibition had been reported previously for PTPs (69).

Our study reveals conformational flexibility in the D1 and D2 linker/spacer region in PTPα and suggests that this flexibility is essential in the regulation of PTPα activity. Our results reveal a novel role for D2 in the direct intramolecular regulation of PTPα activity where we show that it is required for the attainment of a higher activity state. Similarly, the D2 domain of LAR was shown to be required for the stimulation of D1 activity by basic, positively charged proteins and peptides (76). Another example of a D2 domain intramolecularly affecting D1 activity is the case of CD45, in which D2 is required for optimal activity of D1 (25, 77). Interestingly, our results show that although CD45 is a receptor-like PTP with a high degree of homology to LAR and PTPα, CD45 was actually inhibited by many of these compounds rather than activated by them.

Evidence suggests that PTPα and CD45 may be inhibited by intermolecular dimerization of two D1 domains (73, 78–82). In the crystal structure of PTPα-D1, a “wedge” structure in one D1 molecule blocks the active site of another D1 domain (67). Recent data suggest a role for D2 of PTPs in disrupting this dimerization-induced inhibition (69). This study suggests that the spacer region between D1 and D2 and the C-terminal region of PTPα can interact intramolecularly with each other to form a “closed” conformation, which is unable to interact intermolecularly with other PTPα molecules (69). It is possible that
this closed conformation is similar to the basal state proposed in Fig. 9, and the conformational flexibility between the D1-D2 spacer/interdomain region observed in this and an early study (68) may be essential for the regulation of PTPs activity by the D2 domain.

Previous studies have shown that the cytosolic portion of PTPs (such as the constructs used in this study) exists as a monomer in the absence of its membrane-spanning region (65, 85). Although we do not know exactly where the binding site(s) for NSF506 is, NSF506 does not affect the catalytic activity of either PTPα-D1 or PTPα-D2 alone, indicating that both domains are required for PTPα activation by NSF506. Collectively, our data suggest that the potential for potent activation of PTPs by small molecule ligands is an intrinsic property of the PTPα polypeptide chain itself and not simply the result of the relief of intermolecular dimerization-induced inhibition.

Another implication of the potent activation of PTPα by a small molecule ligand is that the activated state may represent a physiologically relevant conformation of this enzyme and thus provides another mechanism by which the substrate specificity of PTPα could be regulated. Other phosphatases such as MKP3 (84) and Cdc25 (85) have been shown to adopt a more active conformation of PTPα-D1 or PTPα-D2 alone, indicating that both domains are required for PTPα activation by NSF506. Collectively, our data suggest that the potential for potent activation of either PTPα-D1 or PTPα-D2 alone, indicating that both domains are required for PTPα activation by NSF506. Collectively, our data suggest that the potential for potent activation of PTPs by small molecule ligands is an intrinsic property of the PTPα polypeptide chain itself and not simply the result of the relief of intermolecular dimerization-induced inhibition.

In conclusion, we have screened 45 suramin analogs for their activity by the pathogenicity of the YopH activity. Two of the receptor-like PTPs used in this study, LAR and PTPα, showed the ability to undergo activation in the presence of some of these small molecules, suggesting a potential regulatory mechanism for these proteins in vivo. We show that the D2 domain is required for this intramolecular activation, revealing a novel function for this domain. Future studies will be needed to address the potential therapeutic utility of these compounds in inhibiting PTPs and the regulation of the receptor-like PTPs.

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