Suramin Is an Active Site-directed, Reversible, and Tight-binding Inhibitor of Protein-tyrosine Phosphatases*

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The effect of suramin, a well known antitrypanosomal drug and a novel experimental agent for the treatment of several cancers, on protein-tyrosine phosphatases (PTPases) has been examined. Suramin is a reversible and competitive PTPase inhibitor with $K_a$ values in the low mM range, whereas the $K_a$ for the dual specificity phosphatase VHR is at least 10-fold higher. Although suramin can also inhibit the activity of the potato acid phosphatase at a slightly higher concentration, it is 2–3 orders of magnitude less effective against the protein Ser/Thr phosphatase 1α and the bovine intestinal alkaline phosphatase. Suramin binds to the active site of PTPases with a binding stoichiometry of 1:1. Furthermore, when suramin is bound to the active site of PTPases, its fluorescence is enhanced approximately by 10-fold. This property has allowed the determination of the binding affinity of suramin for PTPases and several catalytically impaired mutant PTPases by fluorescence titration techniques. Thus, the active site Cys to Ser mutants bind suramin with similar affinity as the wild type, while the active site Arg to Ala mutant exhibits a 20-fold reduced affinity toward suramin. Interestingly, the general acid deficient Asp to Ala mutant PTPases display an enhanced affinity toward suramin, which is in accord with their use as improved “substrate-trapping” agents. That suramin is a high affinity PTPase inhibitor is consistent with the observation that suramin treatment of cancer cell lines leads to an increase in tyrosine phosphorylation of several cellular proteins. Given the pleiotropic effects of suramin on many enzyme systems and growth factor-receptor interactions, the exact in vivo actions of suramin require further detailed structure-activity investigation of suramin and its structural analogs.

Suramin is a polysulfonated naphthylurea compound (see Fig. 1A) that has been widely used for the treatment of trypanosomiasis (sleeping sickness) and onchocerciasis since the early 1920s (1). It was originally synthesized by Bayer AG in 1916 based on the observation that trypan red and trypan blue exhibited trypanocidal activity (2, 3). Since the last decade, many new and therapeutically significant properties of this compound have been identified. For example, suramin has been shown to prevent the infection of T lymphocytes by human immunodeficiency virus in vitro (4). Suramin has also been shown to exert antiproliferative activities by interfering with the binding of a number of growth factors, such as platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, transforming growth factor-β (5–9), and tumor necrosis factor-α (10) to their corresponding receptors. The ability of suramin to block the activity of several growth factors that play important role in tumor cell biology has prompted studies directed at the use of suramin as an antineoplastic agent (3). Indeed, subsequent investigations have shown that suramin exhibits antitumor activity against several metastatic cancers such as renal cancer, adrenocarcinoma, lymphoma, and prostate cancer (11, 12).

If the antitumor property of suramin is solely due to its ability to antagonize the activity of growth factors that stimulate the intrinsic protein-tyrosine kinase activity of the receptors, one would expect a decrease in tyrosine phosphorylation in cancer cells upon exposure to suramin. Unexpectedly, suramin treatment of epidermal carcinoma and several prostate, breast, gastric, and colon cancer cell lines causes rapid and dramatic increase in tyrosine phosphorylation of several cellular proteins (13, 14). These observations cast doubt about the notion that the antitumor action of suramin simply arises from abrogation of growth factor functions and suggest the possibility that suramin may suppress cell growth by altering directly the enzymatic activity of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPases).1 PTPases, in conjunction with protein-tyrosine kinases, control the state of tyrosine phosphorylation in cellular proteins, which regulate a wide variety of biological processes such as cell growth, differentiation and oncogenic transformation (15, 16). Although suramin is capable of reducing the activity of Ser/Thr kinases such as protein kinase C (17, 18) and Cdc2 (19), the effect of suramin on protein-tyrosine kinase activity is unknown. Interestingly, suramin has also been reported to enhance the tyrosine phosphorylation of Cdc2 kinase in a nuclear extract (19) and to inhibit the tyrosine phosphatase activity of a preparation of immunoprecipitated plasma membrane bound CD45 in a noncompetitive and irreversible manner (20).

Given the structural and chemical nature of suramin (Fig. 1A), we were intrigued by the observation that suramin inhibits the CD45 phosphatase activity noncompetitively and irreversibly. Suramin lacks a reactive functionality and possesses six sulfonic acid groups attached directly to aromatic rings. Thus, it is possible that suramin may bind the PTPase active site, which recognize phosphotyrosine (21), and acts as a competitive, reversible PTPase inhibitor. This possibility together with the observation that suramin enhances the tyrosine phosphorylation level of several proteins in tumor cells and in Cdc2

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1 The abbreviations used are: PTPase, protein-tyrosine phosphatases; $\beta$NPP, $\beta$-nitrophenyl phosphate.
prompted us to carry out detailed kinetic and binding studies on homogeneous recombinant *Yersinia* PTPase, the mammalian PTP1B, and several active site-directed mutant PTPases in order to define the mode of action of suramin on PTPases. Furthermore, we have also studied the effect of suramin on the reaction catalyzed by the dual specificity phosphatase VHR, the protein Ser/Thr phosphatase 1α, the potato acid phosphatase, and the bovine intestinal alkaline phosphatase.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were obtained from commercial suppliers and used without further purification. p-nitrophenyl phosphate (pNPP) was from Fluka Co. Suramin (hexaammonium sym-bis[m-aminobenzy1-m-amino-p-methylenzy1-t-naphthalamino-4,8,8-trisulfonate] carbamidine, molecular weight = 1429) was a kind gift from Dr. William Doug Figg at the National Cancer Institute, NIH. Sulfosalicylic acid (3-carboxy-4-hydroxybenzenesulfonic acid) was purchased from Fisher. Solutions were prepared using deionized and distilled water. Acid phosphatase (potato) and alkaline phosphatase (bovine intestine) were purchased from Sigma. Protein phosphatase 1α was a generous gift from Dr. Ernest Lee at the University of Miami. Site-directed mutagenesis kit was from Bio-Rad, and DNA sequencing kit from U. S. Biochemical Corp.

**Enzyme Preparation**—The catalytic domains of the *Yersinia* PTPase (Yop51Δ162, residues 163–468) (22), PTP1B (residues 1–321) (23), and the full-length dual specificity phosphatase VHR (24) were used in this study. The catalytic domains of the *Yersinia* PTPase and PTP1B were kinetically indistinguishable from the corresponding full-length native enzymes and were the forms used in x-ray structural studies (25, 26). The recombinant *Yersinia* PTPase, PTP1B, and VHR were expressed in *Escherichia coli* and purified to homogeneity as described previously (22–24). Protein concentration was determined from absorbance measurement at 280 nm using an absorbance coefficient of 0.493 for the *Yersinia* PTPase, 1.24 for PTP1B, and 0.564 for VHR.

**Site-directed Mutants**—Site-directed mutagenesis experiments that converted Asp-356 in the *Yersinia* PTPase to an Ala (*Yersinia* PTP/D356A) and Asp-181 in PTP1B to an Ala (PTP1B/D181A) were performed using the Mutagen- *in vitro* mutagenesis kit from Bio-Rad. The oligonucleotide primers used were as follows: D356A, 5’-ATTGCGCCGCTCAGACCCG-3’, and D181A, 5’-CATGCGCTGCTTTGTGAGTT-3’, where the underlined base indicates the change from the naturally occurring nucleotide. All mutations were confirmed by DNA sequencing.

**PTPases**—The PTPase activity was assayed at 25 °C in a 1.0 cm length of 315 nm at 25 °C. The inhibition constant of suramin was determined for PTP1B, the *Yersinia* PTPase, and PTP1B/VHR, using NPP as substrate. The assay buffer contained 50 mM 3,3-dimethylglutarate, pH 7.0 with a strong ionic strength of 0.15 M adjusted by sodium chloride (buffer A). The reaction was initiated by addition of the enzyme and quenched after 2–3 min by addition of 1 ml of 1N NaOH. The fluorescence intensity of the bound suramin was followed as a function of time for suramin and the proteins at 315 nm and 405 nm was less than 0.1 in the excitation wavelength of 315 nm.

**RESULTS**

**Time Course for Suramin Binding to the PTPases**—A sample of 2 μM suramin in buffer A was first placed in the cuvette at 25 °C as the reference. An aliquot (50 μl) of PTPase stock was then added and mixed with the suramin solution manually. The final protein concentration was 10 μM. The time course of suramin binding to the PTPase was followed by the increase in fluorescence emission at 405 nm with an excitation wavelength of 315 nm.

**Stoichiometry of Suramin Binding to the PTPases**—In these experiments, the total concentration of suramin and the PTPase were kept constant at 20 μM while the ratio of suramin to the PTPase varied. The fluorescence of the mixture was measured as a function of the ratio of suramin to the PTPase at 25 °C in buffer A. The highest fluorescence intensity was observed when the ratio of suramin to protein equaled to the stoichiometry of the complex composition.

**Reversibility of Suramin Binding to the PTPases**—To assess the reversibility of suramin binding to PTPases, the following experiments were performed. Concentrated PTP1B or the *Yersinia* PTPase (40 μM) and suramin (40 μM) was incubated together at 25 °C in buffer A for two hours and its fluorescence was recorded after a 100-fold dilution. A 20 μl aliquot of the diluted enzyme solution was also withdrawn for activity measurement. Then a small volume of concentrated suramin was added to the 100-fold diluted sample so that the final concentration for suramin and the PTPase was 4.0 and 0.4 μM, respectively. The fluorescence of this sample was measured and compared with the control, which was prepared by adding 4.0 μM suramin directly to 0.4 μM fresh PTPase.

**Binding Studies**—To determine the affinity of suramin to the PTPases, the fluorescence intensity of the bound suramin was followed as increasing amount of the PTPases was added to a solution of suramin at 25 °C in buffer A. The excitation wavelength was 315 nm and the fluorescence emission was monitored at 405 nm. The absorbance of both suramin and the proteins at 315 nm and 405 nm was less than 0.1 in the concentration range used for the study. The dissociation constant $K_d$ was then calculated by multiplying a square fit of the data to Equation

$$
\Delta F = \frac{\Delta e}{2} (P_L + L_0 + K_d - \sqrt{P_L + L_0 + K_d^2} - 4P_L) (Eq. 1)
$$

$$
\Delta F = F - F_L, (Eq. 2)
$$

$$
\Delta e = e_c - e_L - e_P (Eq. 3)
$$

$$
P_L = \frac{P_DAV}{V_0 + \Delta V} (Eq. 4)
$$

$$
L_0 = \frac{L_DAV}{V_0 + \Delta V} (Eq. 5)
$$

where $\Delta F$ is the difference between the fluorescence of titrated sample and the fluorescence of the controls. Two controls were used even though they contributed only a small amount to the total fluorescence. One control consisted of suramin sample titrated with the same volume of buffer and the other control consisted of the buffer titrated with the protein stock. $e_c$ represents a constant which is the difference between the fluorescence coefficient of bound suramin and those of free suramin and free protein under the selected excitation and emission wavelength. $V_0$ is initial volume of the solution in the cuvette and $\Delta V$ is the volume of the titrating agent. $P_D$ is the protein concentration of stock solution, and $L_0$ is the initial suramin concentration in the cuvette.

**Effect of Vanadate on Suramin Binding**—One mM vanadate was added to the mixture of suramin (2 μM) and PTP1B (10 μM) or the *Yersinia* PTPase (10 μM). Suramin fluorescence of this sample was measured and compared with the fluorescence of the sample in absence of vanadate.

**Data Presentation**—All of the experiments were reproducible and were carried out in duplicate or triplicate. Each set of experiments were repeated at least three times with similar results. The details for data analysis is described above and the results are shown as means ± standard error.

**RESULTS**

**Suramin Is a Tight-binding and Competitive Inhibitor of PTPases**—Table I summarizes the steady state kinetic parameters for the reaction catalyzed by PTP1B, the *Yersinia* PTPase, and the dual specificity phosphatase VHR, using pNPP as a substrate at pH 7 and 25 °C. PTPases utilize an invariant Asp residue (Asp-181 in PTP1B and Asp-356 in the *Yersinia* PTPase) as a general acid/base to assist phosphate
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TABLE I
Kinetic parameters of PTPases with pNPP as a substrate at 25 °C, pH 7.0

| PTP1B             |  | Yersinia PTP |  | VHR            |  |
|-------------------|---|--------------|---|----------------|---|
|                   | $K_{m}$ | $k_{cat}$ | $K_{m}$ | $k_{cat}$ | $K_{m}$ | $k_{cat}$ |
| Wild type         | 2.4 ± 0.2 | 9.8 ± 0.5 | 1.3 ± 0.3 | 35 ± 2 | 9.3 ± 0.6 | 2.4 ± 0.1 |
| D181A             | 0.08 ± 0.03 | 0.031 ± 0.03 | 0.10 ± 0.01 | 0.04 ± 0.01 | 26 ± 5 | 0.003 ± 0.0002 |

Monoester hydrolysis (30–33). Replacement of Asp181 in PTP1B or Asp-356 in the Yersinia PTPase by an Ala reduced the $k_{cat}$ value by 300- and 900-fold, respectively. Interestingly, substitution of Asp-181 in PTP1B or Asp-356 in Yersinia PTPase with an Ala improved the $K_{m}$ for pNPP by 30- and 10-fold, respectively. Arg-409 of the Yersinia PTPase is a key residue in the PTPase signature motif (28). The guanidinium group in Arg-409 makes a bidentate hydrogen bond with the phosphoryl moiety of the substrate (26) and is involved in both substrate binding and catalysis (28). The $K_{m}$ value of the R409A mutant Yersinia PTPase increased by 20-fold while its $k_{cat}$ was reduced by 10,000-fold.

We have evaluated the ability of suramin to inhibit the action of PTPases and investigated the mode of inhibition by suramin at 25 °C, pH 7.0. As shown in Fig. 2A, the effect of suramin on the PTP1B-catalyzed pNPP hydrolysis displayed the characteristic intersecting line pattern for competitive inhibition. Similar results were obtained for the Yersinia PTPase and the dual specificity phosphatase VHR. Thus, suramin acts as a competitive inhibitor for PTP1B, the Yersinia PTPase and VHR. The competitive inhibition pattern is not surprising considering the structural similarity of aryl sulfonate moieties in suramin with phosphotyrosine. The $K_{m}$ values for suramin with the Yersinia PTPase and PTP1B are 1.3 and 4.0 μM, respectively, while the $K_{m}$ for suramin with VHR is 48 μM, which is more than 10 times greater than those for the Yersinia PTPase and PTP1B (Table II). We also tested the ability of a simple aryl sulfonic acid derivative, sulfosalicylic acid (Fig. 1B), to inhibit PTPases. As expected, sulfosalicylic acid inhibited the Yersinia PTPase activity competitively (Fig. 2B), with a $K_{i}$ value of 7.5 ± 0.2 mM.

Effects of Suramin on Other Phosphatases—Is the inhibition by suramin on PTPases specific? To answer this question, we have determined the effect of suramin on pNPP hydrolysis catalyzed by potato acid phosphatase, bovine intestinal alkaline phosphatase, and protein Ser/Thr phosphatase 1α. Suramin inhibited the potato acid phosphatase-catalyzed reaction and the pattern of inhibition was noncompetitive, with $K_{i} = 9.3 ± 0.9$ μM and $K_{i} = 11.2 ± 0.6$ μM, respectively. The pattern of inhibition for suramin in the protein phosphatase 1α-catalyzed reaction was also noncompetitive, but in this case, $K_{i} = 250 ± 90$ μM and $K_{i} = 220 ± 60$ μM. Interestingly, suramin acted as a competitive inhibitor for the alkaline phosphatase-catalyzed reaction, with a $K_{i}$ value of 1.4 ± 0.1 mM.

Changes of Suramin Fluorescence upon Interaction with PTPases—Free suramin displayed a low fluorescence intensity when excited at 315 nm (34). The fluorescence spectra of suramin in the presence of increasing concentrations of PTP1B showed a pronounced increase in the emission intensity at the fluorescence maximum whereas the maximum emission wavelength ($\lambda_{max} = 405$ nm) of suramin did not change2 (Fig. 3).

Similar observations were also made for the Yersinia PTPase and VHR. The fluorescence enhancement was 10-fold for PTP1B at the ligand and enzyme concentration of 4 and 16 μM, respectively. Such a fluorescence enhancement of suramin upon the binding of PTP1B can be used to measure the affinity of suramin for PTPases using techniques of fluorescence titration.

Suramin Binding to PTP1B and the Yersinia PTPase Is Rapid and Reversible—PTPase inhibition experiments indicate that suramin is a high affinity inhibitor for PTP1B and the Yersinia PTPase. In order to measure the binding constant by fluorescence titration, a fast and reversible binding is required. The fluorescence intensity of free suramin was low. When PTP1B was added to suramin, its fluorescence increased rapidly and reached 90% of its maximum within 30 s (which was the time required for the manual mixing, data not shown). The fluorescence then increased gradually and reached the maximum in 10 min. A similar time course was observed upon suramin binding to the Yersinia PTPase. These results indicate that suramin binding to PTPases is a fast process.

To assess the reversibility of the binding of suramin to the PTPases, the phosphatase activity and suramin fluorescence of the native PTPases were compared with those of PTPases released from the suramin bound complexes. PTP1B (40 μM) was first mixed with an equal amount of suramin, which should result in 73% of PTP1B in the suramin-bound form (this estimate was based on the $K_{i}$ of suramin of $\sim 4$ μM for PTP1B). When the sample was diluted by 100-fold, more than 90% of PTP1B should be in the ligand-free form. The fluorescence spectrum of the diluted solution containing 0.4 μM suramin and 0.4 μM PTP1B is shown in Fig. 4 (spectrum 2). Suramin (4 μM) alone had little fluorescence (Fig. 4, spectrum 1). However, when 3.6 μM of suramin was added to the diluted PTP1B and suramin sample (Fig. 4, spectrum 3), the fluorescence increased and reached to the same level as the control (Fig. 4, spectrum 4), which was made of freshly mixed PTP1B (0.4 μM) and suramin (4.0 μM). This result suggests that PTP1B released from the PTP1B-suramin complex still has the native conformation and is able to bind suramin again. This conclusion is supported by measurements of the phosphatase activity of PTP1B released from the preformed PTP1B-suramin complex. When the 100-fold diluted PTP1B and suramin mixture was diluted 10-fold further for activity measurement, the enzyme activity of this 1,000-fold diluted sample was found to be exactly the same as the native PTP1B in the absence of suramin. This indicates that PTP1B released from the suramin-bound form possess full enzymatic activity. Similar results were also obtained with the Yersinia PTPase. Thus, the interaction between suramin and PTPases is rapid and reversible.

Stoichiometry and Site of Interaction for Suramin Binding—Suramin fluorescence increases when it binds to PTPases. To determine the stoichiometry of suramin binding to PTPases, the dependence of the fluorescence intensity of the PTPase-

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2 The maximum fluorescence emission wavelength of suramin did not change during titration with PTP1B. Similar observation has been made when neutrophil serine proteinases were added to a suramin solution (34). The slight increase in fluorescence at 380 nm in the spectra at high PTP1B concentrations was due to the small intrinsic fluorescence of PTP1B, which peaks at 340 nm (excitation wavelength 315 nm). In the fluorescence titration experiments, the contribution from the protein to the total fluorescence was corrected by subtraction (see “Experimental Procedures”).
suramin mixture on the ratio of suramin to the protein was measured, while keeping the total concentration of suramin and the protein constant. Fig. 5 shows that suramin fluorescence reaches to the maximum at a suramin to PTP1B ratio of 1:1. A stoichiometry of 1:1 was also observed for suramin binding to the Yersinia PTPase, VHR and all of the mutants PTPases examined, including PTP1B/D181A, PTP1B/C215S, Yersinia PTP/D356A, Yersinia PTP/C403S and Yersinia PTP/R409A. These results indicate that these PTPases and their mutants possess only one binding site for suramin.

Since suramin displays competitive inhibition pattern against these PTPases, it is important to determine whether the suramin binding site on the PTPases corresponds to the PTPase active site (i.e. the phosphotyrosine binding site). Vanadate is a well known competitive inhibitor of PTPases and the crystal structure of the Yersinia PTPase complexed with vanadate reveals that vanadate binds at the active site (35). When 1 mM of vanadate was added to the solution containing both suramin and PTP1B (spectrum 1 in Fig. 6), the suramin fluorescence decreased dramatically (spectrum 2 in Fig. 6). The dose dependence for the ability of vanadate to suppress binding of suramin to the active site of PTP1B is shown in the inset of Fig. 6. These results indicated that vanadate was able to displace suramin from the active site of PTP1B. Similar observation was also made for the Yersinia PTPase. Furthermore, an active site mutant PTPase with decreased affinity for substrates and oxyanions also displays a reduced binding toward suramin (see below). Collectively, all of the data suggest that suramin, like vanadate, binds at the active site of PTPases.

**Binding Constants for the Association of Suramin with PTPases Determined by Fluorescence Titration**—We have shown that suramin binds to the active site of PTPases rapidly and reversibly. The PTPase-bound suramin exhibits enhanced fluorescence emission at 405 nm when excited at 315 nm. These desirable properties have allowed us to determine the suramin binding constants for PTP1B, the Yersinia PTPase and several of the active site-directed mutants PTPases with low or no catalytic activities. A typical fluorescence titration curve for the determination of binding constant of suramin to PTP1B is shown in the inset of Fig. 3. The solid line was obtained by a nonlinear least square fit of the titration data to the 1:1 binding model as described under “Experimental Procedures.” The dissociation constants for suramin binding to PTP1B, the Yersinia PTPase, and the mutants are listed in Table II. The $K_d$ values determined by fluorescence titration for PTP1B and Yersinia PTPase were 1.4 and 3 $\mu M$, respectively, which were similar to the inhibition constants measured by steady state kinetics. The slight differences in the results between fluorescence titration and inhibition study might be caused by the differences in experimental conditions, such as enzyme concentrations.

| PTP1B         | $K_a$ | $K_d$ | Yersinia PTP | $K_a$ | $K_d$ | VHR  | $K_a$ |
|---------------|-------|-------|--------------|-------|-------|------|-------|
| Wild type     | 4 ± 1 | 1.4 ± 0.2 | Wild type    | 1.3 ± 0.3 | 3.0 ± 0.6 | Wild type | 48 ± 10 |
| D181A         | 0.24 ± 0.05 | 0.24 ± 0.05 | D356A        | 0.6 ± 0.1 | 0.6 ± 0.1 | D356A | 0.6 ± 0.1 |
| C215S         | 3.0 ± 1.0 | 3.0 ± 1.0 | C403S        | 2.1 ± 0.4 | 2.1 ± 0.4 | C403S | 2.1 ± 0.4 |
| R409A         | 58 ± 14 | 58 ± 14 | R409A        | 14 ± 0.6 | 14 ± 0.6 | R409A | 14 ± 0.6 |

* Determined by inhibition experiments.

b Determined by fluorescence titration experiments.
The general acid deficient mutant PTPases (PTP1B/D181A and Yersinia PTP/D356A) and the active site Arg mutant (Yersinia PTP/R409A) display very low phosphatase activity. Furthermore, the active site Cys to Ser mutant PTPases (PTP1B/C215S and Yersinia PTP/C403S) possess no measurable phosphatase activity at all (27). Thus, direct measurements of the affinity of suramin toward these mutant PTPases by enzyme kinetic inhibition experiments can be difficult. The described fluorescence titration is an ideal method to measure suramin binding constants for these low activity PTPase mutants. Interestingly, both PTP1B/D181A and Yersinia PTP/D356A displayed higher affinity (5-fold) than the wild type enzyme toward suramin. Substitution of Cys-215 in PTP1B and Cys-403 in the Yersinia PTPase by a Ser did not change the PTPase’s affinity for suramin, even though the phosphatase activity was completely abolished. The guanidinium side chain of Arg-409 in the Yersinia PTPase interacts with two oxygens in the phosphoryl moiety of a substrate or an oxyanion (26). Substitution of Arg-409 by an Ala in the Yersinia PTPase has been shown to decrease the affinity of the Yersinia PTPase toward pNPP (a substrate) and arsenate (an oxyanion competitive inhibitor) by 30- and 40-fold, respectively (28). Here we show that replacement of Arg-409 by an Ala in the Yersinia PTPase also resulted in a 20-fold decrease in the phosphatase’s affinity toward suramin. These results suggest that the active site Arg residue interacts with the oxygens on the sulfonate group of suramin in the same manner as it interacts with the oxygens on the phosphoryl moiety, and is consistent with the conclusion that suramin binds the PTPase active site.

**DISCUSSION**

We have examined the effect of suramin on two representative PTPases, human PTP1B and the Yersinia PTPase, both of which have been extensively characterized biochemically and structurally (36). Included in the PTPase superfamily is a subfamily of enzymes called dual specificity phosphatases that can catalyze the hydrolysis of not only phosphotyrosine but also phosphoserine/threonine (37). The effect of suramin on the human dual specificity phosphatase VHR, the best characterized member of this family of phosphatases, is also investigated. Using a variety of kinetic and fluorescence titration techniques, we show that suramin is indeed a high affinity PTPase inhibitor. In addition, we demonstrate that the mode of suramin inhibition is competitive and that the binding of suramin to the PTPases and VHR is rapid and reversible. Finally, we conclude that the binding stoichiometry of suramin to PTPases is 1:1 and suramin binds directly to the active site of the PTPases and VHR. These results are in direct contrast to a previous report that suramin inhibits the phosphatase activity of the receptor-like PTPase CD45 noncompetitively and irreversibly (20). We do not know the source for this discrepancy.

We note that, in the early work, an immunoprecipitated...
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FIG. 6. Effect of vanadate on the binding of suramin to PTP1B at pH 7.0 and 25°C. Spectrum 1, suramin at a concentration of 2 μM in the presence of 10 μM PTP1B; spectrum 2, the mixture of 2 μM suramin, 10 μM PTP1B, and 1 mM vanadate; and spectrum 3, suramin alone at concentration of 2 μM. The inset shows the dose dependence for the ability of vanadate to displace suramin (2 μM) from PTP1B (20 μM). The data were fitted to the following equation by a nonlinear regression analysis

\[
\frac{\Delta F}{\Delta F_0} = \frac{1 + K_d^V}{2-K_d^V} \left(1 - \frac{C^V}{C^V + C^s} - \frac{[k^V]_0}{[k^V]_0 + [s]^2}\right)^2 - 4 \cdot C^V \cdot K_d^V
\]

where vanadate and suramin are assumed to bind PTP1B competitively, and suramin concentration is much smaller than the enzyme concentration. \(\Delta F\) is the fluorescence of the sample in the presence of vanadate and \(\Delta F_0\) is the fluorescence of the sample without vanadate. \(C^V\) is the enzyme concentration which is fixed at 20 μM, \(C^s\) is the vanadate concentration during titration, \(K_d^V\) is the dissociation constant for the suramin-PTP1B complex with a value of 1.4 μM (Table 1), and \(K_d^s\) is the dissociation constant for the vanadate-PTP1B complex. By fitting the dose-dependence curve, a dissociation constant (\(K_d^V\)) of 15 ± 1 μM was obtained for the PTP1B-vanadate complex. This value is 30-fold higher than the \(K_d^s\) (0.52 ± 0.05 μM) for vanadate determined under steady state conditions in the PTP1B reaction. This apparent discrepancy may result from differences in enzyme concentrations in the two experiments or that the presence of suramin may decrease the affinity of vanadate to PTP1B.

In the presence of 10 μM PTP1B, 2 μM suramin, and 1 mM vanadate, the affinity of VHR for suramin is 10–40-fold lower than those of the PTPases.

To answer the question whether suramin displays a special affinity for PTPases, we also examined the ability of suramin to inhibit pNPP hydrolysis catalyzed by the nonspecific acid and alkaline phosphatases as well as protein phosphatase 1α. At the pH optima for activity measurement, suramin inhibits the potato acid phosphatase-catalyzed reaction noncompetitively, with dissociation constants severalfold higher than those of the PTPases, whereas suramin is not an effective inhibitor for protein phosphatase 1α and alkaline phosphatase, with dissociation constants 2 and 3 orders of magnitude higher than those of the PTPases. Thus it does not appear that suramin inhibit phosphatases nondiscriminately.

We have also established that sulfosaliclylic acid is a competitive inhibitor for the Yersinia PTPase. However, the affinity of sulfosalicylic acid for the Yersinia PTPase is 5,700-fold lower than that of suramin. This implies that structural features in addition to the aryl sulfonate motif in suramin are required for high affinity binding. It is known that the phosphotyrosine binding site (the active site) of PTP1B possesses significant plasticity so that substituted naphthalene derivatives containing a difluoromethyleneephosphonyl group can also be accommodated (39). Thus, the polysubstituted sulfonyl naphthalene moiety may be responsible for the enhanced affinity of suramin for PTPases. It is possible that the additional polysubstituted sulfonyl naphthalene moiety and/or other functionalities in suramin are also important for the high affinity binding.

When suramin is bound to the active site of PTPases, its fluorescence is enhanced approximately 10-fold. This property makes suramin a valuable tool to study the ligand binding reaction for the catalytically impaired PTPase mutants. The active site Cys residue in PTPases acts as a nucleophile to attack the phosphorous atom on the substrate (40, 41). Although replacing the Cys residue with a Ser residue abolishes the PTPase activity (27), the mutant protein can still bind substrates, i.e. Tyr(P)-containing peptides/proteins (42–44). Consistent with these observations, our work shows that PTP1B/C215S and Yersinia PTP/C403S retain similar affinity for suramin as the wild type enzymes. Elimination of the guanidinium side chain of Arg-409 in the Yersinia PTPase, a residue important for the binding of the phosphatase group in a substrate, reduces the affinity of the PTPase for suramin by 20-fold, which is consistent with the conclusion that suramin binds to the PTPase active site. When the invariant catalytic Asp residue is changed to an Ala, the phosphatase activity of the general acid/base-deficient PTPases is reduced dramatically. Interestingly, the affinity of PTP1B/D181A and Yersinia PTP/D356A for suramin increases by 5–30-fold. This is consistent with the observation that the general acid-deficient mutant PTPase is a better “substrate-trapping” reagent than the active site Cys to Ser mutant for the identification of physiological PTPase substrates in vitro (45).

Suramin is being evaluated in phase II and III clinical trials for the treatment of prostate cancer and other solid tumors. It is believed that the beneficial effects of suramin treatment derive from its ability to block the activity of a number of growth factors. The concentration of suramin required for maximal effect was within clinically achievable and tolerable plasma suramin levels (300 μg/ml) (12). This concentration (200 μM) of suramin is 2 orders of magnitude higher than that required to shut down the in vitro activity of PTPases, which play important roles in regulating signal transduction processes initiated by growth factors. This large difference between the therapeutically effective serum suramin concentration and the concentration required to inhibit PTPases in vitro...
may not be too surprising for the following reasons. It is known that suramin is highly bound to plasma proteins such as albumin (46, 47), which may reduce the effective free suramin concentration in circulation. Furthermore, because of its charge, suramin is membrane impermeable and has to be actively transported into cells (12). Thus, the concentration of suramin inside the cell may well be much lower than the therapeutic suramin concentration in the serum. It is possible that the increase in tyrosine phosphorylation in cellular processes may come from the inhibition of PTPases.

In addition to the growth factors and PTPases, suramin also exerts pleiotropic effects on a variety of enzyme systems. For example, within the concentration range between 1 and 100 μM, suramin can inhibit the activity of protein kinase C (17, 18) Cdc2 (19), phosphatidylinositol kinase and diacylglycerol kinase (48), DNA and RNA polymerase (49, 50), reverse transcriptase (51), DNA topoisomerase (52), steroid 5α-reductase (53), ATPase (54), and neutrophil serine proteinases (34). Clearly, a detailed understanding of the effects of suramin and which of these effects are important for the antitumor therapeutic suramin concentration in the serum. It is possible that suramin inside the cell may well be much lower than the concentration of suramin can inhibit the activity of protein kinase C (17, 18) Cdc2 (19), phosphatidylinositol kinase and diacylglycerol kinase (48), DNA and RNA polymerase (49, 50), reverse transcriptase (51), DNA topoisomerase (52), steroid 5α-reductase (53), ATPase (54), and neutrophil serine proteinases (34). Clearly, a detailed understanding of the effects of suramin in vivo and which of these effects are important for the antitumor activity requires further systematic structure-activity studies of suramin and its structural analogs.

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