

**SURAMIN, AN EXPERIMENTAL CHEMOTHERAPEUTIC DRUG, IRREVERSIBLY
BLOCKS T CELL CD45-PROTEIN TYROSINE PHOSPHATASE IN VITRO**

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SUMMARY: Suramin has long been used for the treatment of Gambian and Rhodesian trypanosomiasis and oncocerciasis. More recently, the demonstration that suramin inhibits DNA polymerases, reverse transcriptase and the lymphocyte terminal deoxynucleotidyl transferase has led to its clinical trials for the treatment of AIDS and cancer. The precise nature of suramin's anti-neoplastic action is not clear at this time. Suramin rapidly alters the tyrosine - specific phosphorylation of cellular proteins in many cancer cell lines. Here we demonstrate that suramin strongly inhibits the activity of CD45, the principal tyrosine specific protein phosphatase of T lymphocytes. Suramin-induced inactivation of CD45 is noncompetitive, irreversible and complete within 10 min. The ability of suramin to block CD45 mediated phosphatase function provides both new insight into the mechanism of action of this agent and a useful new probe for studies of T cell activation. © 1993 Academic Press, Inc.

Protein tyrosine phosphorylation and dephosphorylation regulate receptor activity, cell proliferation and malignant transformation (1, 2). CD45, also known as leukocyte common antigen (LCA), Ly5, or T200, comprises a class of high molecular weight, single chain, transmembrane protein tyrosine phosphatases abundantly expressed on all nucleated cells of hematopoietic origin (3). Within T cells, CD45-mediated dephosphorylation of Tyr-505 of the tyrosine-specific src-related protein kinase (TPK) p56^{lck}, converts this kinase to its active form (4). Phosphorylation of p56^{lck} at Tyr-505 by another TPK, p50^{ck}, has been shown to inactivate p56^{lck} kinase function (5). Studies of CD45 negative cell lines have shown that expression of CD45 is essential for antigen induced signal transduction, IL2 production and proliferation (6-8).

Suramin, a polyanionic drug, has been widely used for the treatment of human and bovine trypanosomiasis since its introduction in the early 1920s (9). Although suramin forms stable complexes with a variety of proteins and inhibits a number of enzyme systems, its mechanism of action is still unclear. Suramin is not a general cytopathic agent (10), and does not penetrate erythrocytes to cause damage directly to intracellular parasites (9). Suramin does, however, inhibit with high potency the in vitro growth of many tumor cell lines, particularly those of lymphoid origin (10), and for this reason the drug has recently been used experimentally as an anti-cancer agent (11-14). A number of biochemical mechanisms have been proposed to explain suramin's effect on cell growth. Suramin has been found to be a competitive inhibitor of retroviral

reverse transcriptase (15), can protect T cells in vitro against HIV infection and cytopathic effect at doses that are clinically plausible (16), and can cause the reversion of the anaplastic phenotype of human and rat fibroblasts transformed by simian sarcoma viruses (17).

In normal mice suramin induces profound and prolonged thymic involution and splenic lymphopenia, suggesting that lymphocytes may be particularly susceptible to suramin toxicity (10). In clinical trials, doses of suramin that inhibit viral replication in human patients were found to be immunosuppressive (18). Suramin seems to interfere with the binding of a number of polypeptide growth factors, including PDGF, FGF, TGF β , and IL-2 to their respective cell surface receptors (19-22), and it is possible that such interference may contribute to its immunosuppressive effects. The high doses of suramin needed to inhibit IL-2/IL-2R binding, and the ability of IL-2 to overcome much of the suramin-mediated growth inhibition suggest, however, that additional mechanisms may be involved in the drug's anti-immune properties. Suramin itself is a chemotactic signal for monocytes and it enhances in vitro phagocytic activity of monocytes (measured by the phagocytic uptake of yeasts) (23).

A recent report (24) has shown that suramin at a dose of 100 μ M (= 143 μ g/ml) can dramatically increase tyrosine-specific phosphorylation of cellular proteins in the epidermal carcinoma cell lines A431 and KB. Moreover, suramin causes a remarkable and rapid increase in the tyrosine specific phosphorylation of proteins in several prostate (PC3, DU145, PC3M, LNCaP, etc.) and colon (HT29, WiDR, SW620, COLO205, etc.) cancer cell lines (25). These observations are consistent with the idea that suramin could alter the activation and growth through an effect on the network of tyrosine-specific protein kinases and phosphatases. We show here that suramin, at doses as low as 10 μ M, is a potent, irreversible, noncompetitive inhibitor of CD45, the principal tyrosine protein phosphatase of T lymphocytes.

MATERIALS AND METHODS

Mice: Mice used for these experiments were either B6D2F1 animals purchased from the Jackson Laboratories, or in some cases genetically heterogeneous mice produced by a four-way cross using SJL/J, AKR/J, C57BL/6J and DBA/2J grandparents. They were housed under specific-pathogen free conditions, given access to food and water ad libitum, and euthanized by CO₂ asphyxiation when 2-4 months of age. Male and female mice were both used.

Materials: Rabbit anti-mouse immunoglobulin antibody for depletion of Ig⁺ cells was purchased from ICN Biomedicals (Costa Mesa, CA). Cells of the M1/9.3.4.HL2 (TIB122) hybridoma line, which produce rat IgG2a antibody to mouse CD45, were purchased from the American Type Culture Collection (Rockville, MD); this antibody recognizes CD45 molecules without regard to differentially spliced exons A, B, and C (26). Nonimmune rat IgG2a was purchased from Zymed Immunochemicals (South San Francisco, CA). Agarose-conjugated antibody to rat IgG was purchased from Sigma Chemical Company (St. Louis, MO). Suramin was the kind gift of Carl Huntley and the NCI Clinical Repository (Rockville, MD). Orthovanadate was purchased from Fischer Scientific (Fair Lawn, NJ), and all other reagents from Sigma Chemical Co., St. Louis, MO.

Preparation of T cells: Spleens were removed aseptically and a single cell suspension prepared by rubbing the tissue between two frosted glass slides into Hanks' balanced salt solution supplemented with 0.2 % bovine serum albumin (HBSS-BSA). The cell suspension was filtered through nylon mesh, washed twice in HBSS-BSA, and layered over Lympholyte-M (Cedarlane Laboratories, Ontario, Canada) to remove erythrocytes. Mononuclear cells were collected from the interface and washed twice. B cells were then depleted by a plate adherence method (27) by panning over rabbit anti-mouse Ig coated plates (negative selection). The resulting cell suspension routinely contained 80-85% T cells as assessed by flow cytometry using anti-Thy-1.2 antibodies.

Preparation of plasma membrane: Spleen T cells (approx. 10^8 cells) were isolated from pools of 4 young adult mice following Lympholyte-M separation and negative selection by panning. Cells were washed with buffered saline (150 mM NaCl containing 20 mM Tris-Cl, pH 7.5). The cell pellet was suspended in 2 ml hypotonic buffer (25 mM Tris-Cl, pH 7.5, 25 mM sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF and 10 μ g/ml leupeptin) and homogenized 20 times in a Dounce type homogenizer. The lysate was centrifuged for 5 minutes at low speed and the supernatant was saved. The pellet was resuspended in 2 ml hypotonic buffer and the process was repeated. Plasma membranes were pelleted down from the combined supernatants by centrifugation at 37784 x g for 45 mins at 4°C in a Beckman table top ultracentrifuge (Model: Optima TL). The membrane pellet was suspended in 500 μ l of hypotonic buffer and used for enzymatic assay.

Immunoprecipitation: T cells (approximately 25×10^6 /ml) were lysed in 1% NP-40 in TN buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 3 mg/ml DTT) for 1 hour at 4°C with occasional gentle shaking. The lysate was centrifuged at 14470 x g for 15 min and the supernatant then precleared with 25 μ l of anti-rat IgG-agarose (Sigma) for 1 hour at 4°C using a tube rotator. The agarose conjugates were pelleted down at 2000 rpm for 1 min in a Beckman microfuge (Model-11). CD45 molecules were then immunoprecipitated from the supernatant by treating with anti-CD45 monoclonal antibody (clone M1/9.3.4.HL2) at a dose of 2.5 μ g/ml (0.1 μ g/ 10^6 cells), followed by incubation with anti-rat IgG-agarose (40 μ l/ml) for 1 hour at 4°C on a tube rotator. The CD45-agarose conjugates were then pelleted at 2000 rpm for 1 min in the microfuge at 4°C. The precipitates were washed two times with 0.5 % NP-40 in TN, two times with TN alone and once with PTPase buffer (100 mM Sodium acetate, pH 6.0, 1 mM EDTA, 0.1 % Triton X-100 and 0.1 % 2-mercaptoethanol) and suspended in 250 μ l PTPase buffer. Control samples were mock-immunoprecipitated using normal rat IgG2a in the same protocol.

PTPase assay using pNPP: CD45-PTPase was assayed in a 50 μ l volume using para-nitrophenyl phosphate (pNPP) as substrate. The complete assay mixture in a total volume of 50 μ l contained 100 mM Sodium acetate, pH 6.0, 1 mM EDTA, 0.1 % Triton X-100, 0.1 % 2-mercaptoethanol and 10 mM pNPP. After 10 min incubation at 37°C with occasional gentle agitation, 100 μ l of 0.25(N) NaOH was added to stop the reaction; the reaction tubes were then vortexed and microfuged at 2000 rpm for 1 min. Absorbance of para-nitrophenol was measured in a 125 μ l aliquot using a microplate reader at 410 nm. Each experiment included control tubes in which addition of immunoprecipitate was delayed until after the addition of NaOH.

PTPase assay using O-phosphotyrosine: In some experiments, as indicated in the text, PTPase activity was determined using O-phosphotyrosine as substrate according to the method of Mustelin et al. (28). The reactions were set up in a total volume of 100 μ l, and stopped after 10 min at 37°C by addition of 100 μ l of 25% TCA followed by 50 μ l of 10% BSA. Precipitates were removed by centrifugation (12000 x g for 4 mins) and the supernatants were used for measurement of liberated phosphate by the method of Chen et al. (29) using a mixture of sulfuric acid, ammonium molybdate and ascorbic acid. The mixtures were incubated at 37°C for 1 hour and the absorbance measured at 750 nm using KH_2PO_4 as standard.

RESULTS

Figure 1 shows the inhibition of membrane bound PTPase activity by varying concentrations of suramin using pNPP as substrate. Okadaic acid and calyculin A (20 nM each) were used as inhibitors of PP2A and PP1. This PTPase activity was 95% inhibited by 1 mM vanadate (data not shown).

Figure 2 shows the results of a set of preliminary experiments to characterize PTPase activity, using pNPP as substrate, in CD45 immunoprecipitated from T cell lysates. Figure 2a shows that hydrolysis of pNPP proceeded linearly up to at least 12 min of incubation. Figure 2b demonstrates the pH dependence for this reaction, which was optimal in slightly acidic conditions, consistent with previous reports (28). We also examined the effects of vanadate, shown in other systems (30, 31) to be a potent inhibitor of PTPase function. Figure 2c shows that PTPase was inhibited by Na_3VO_4 in a dose dependent manner, with half maximal inhibition at 100 μ M.

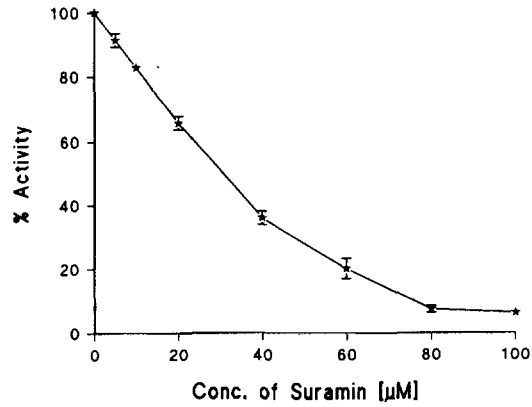


Fig. 1. Dose dependent inhibition of membrane bound PTPase activity by suramin. T cell plasma membrane PTPase activity was measured using pNPP as substrate. Membrane fractions were treated with suramin at room temperature for 10 min and the reactions were started with the addition of 10 mM pNPP. Okadaic acid and calyculin A (20 nM each) were present as inhibitors of PP2A and PP1.

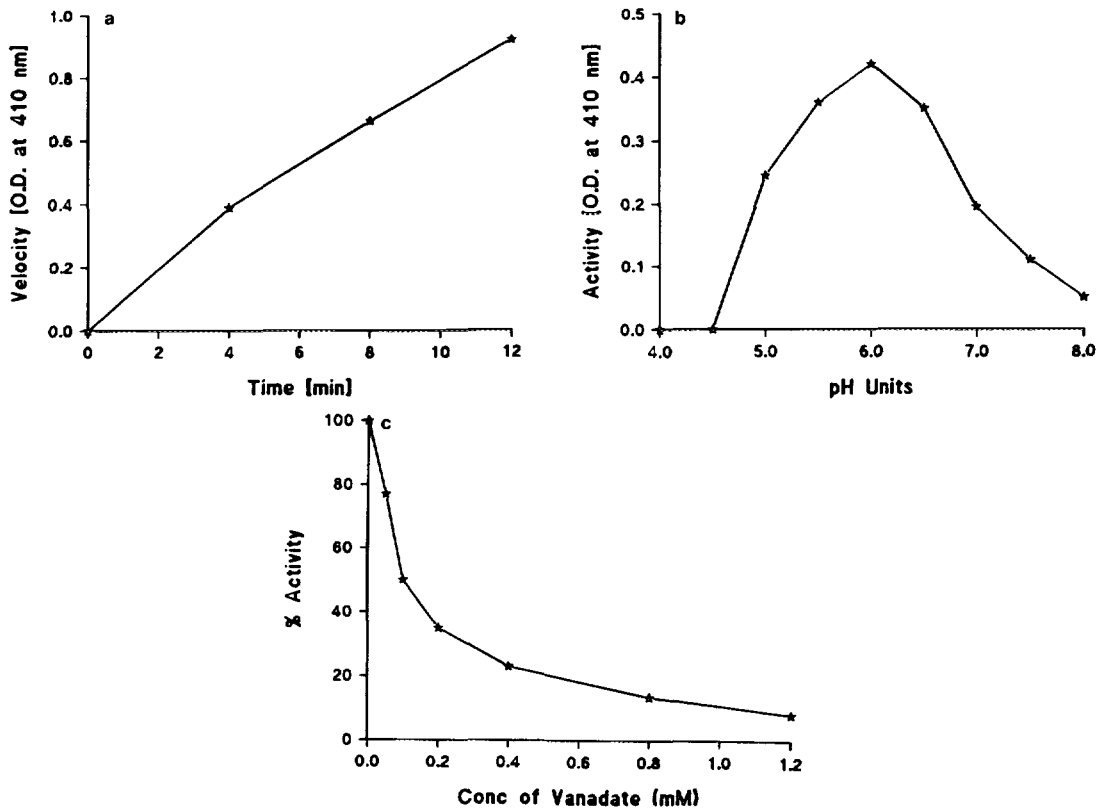


Fig. 2. Characterization of CD45 phosphatase activity in immunoprecipitates from mouse T lymphocytes, using pNPP as substrate. Panel a: Time course at pH 6.0. Panel b: pH profile, 10 min assay. Buffer used for the assay were 100 mM sodium acetate, pH 4.0-6.0, 100 mM imidazole, pH 6.5-8.0. Panel c: Inhibition of CD45 phosphatase activity by Na_2VO_4 in a dose dependent manner, with half maximal inhibition at 100 μM .

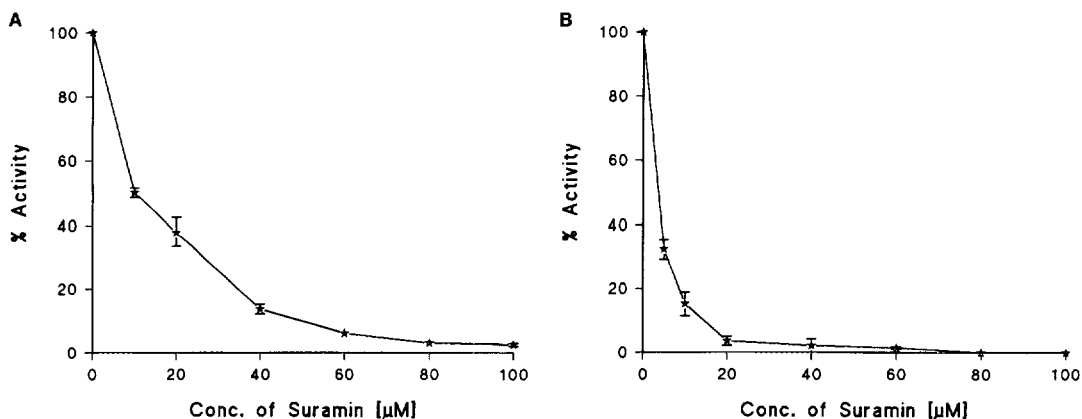


Fig. 3. Inhibition of CD45-PTPase activity by suramin. Immunoprecipitates were preincubated with suramin at the indicated concentrations for 10 min at 37°C prior to assay. Panel A: 10 mM pNPP as substrate. Panel B: 10 mM O-phosphotyrosine as substrate.

Immunoprecipitates using an isotype-matched control antibody did not show any detectable PTPase activity (not shown).

A second series of experiments was carried out using O-phosphotyrosine as substrate. The immunoprecipitates showed good specificity for O-phosphotyrosine, and failed to release phosphate from O-phosphoserine or O-phosphothreonine (data not shown).

Figure 3 shows that suramin strongly inhibits phosphatase function in CD45 immunoprecipitates using either pNPP (panel A) or O-phosphotyrosine (panel B) as substrate. Half maximal inhibition was typically observed at suramin concentrations of 5 - 10 μM (equivalent to 7 - 14 μg/ml), i.e. well below the concentrations that are attained clinically in suramin-treated patients, whose serum levels are typically 200 - 300 μg/ml (11, 12).

Suramin-mediated inhibition of CD45 phosphatase activity is rapid and irreversible. Figure 4 shows data from an experiment in which CD45 immunoprecipitates were incubated with varying doses of suramin for 10 min, and then washed four times before PTPase assay. Washed preparations of suramin-treated CD45 (Figure 4) were as inactive as preparations (Figure 3A) in which equivalent doses of suramin were present during the PTPase assay. The time course for inactivation is shown in Figure 5. At 80 μM suramin, as little as 2 min of incubation at 37°C was sufficient to produce 50% inactivation, and the inactivation was essentially complete within 5 - 10 min.

Figure 6 shows substrate vs velocity curves for CD45 PTPase activity in absence and presence of 20 μM suramin; the inset shows double reciprocal plot of the same data. Suramin induced inhibition was not overcome by increasing substrate concentration. Maximum velocity was not achieved even with very high (20 mM) substrate levels. These results show that suramin is not competing with the substrate for the active site of the phosphatase.

Table 1 summarizes a series of analyses of other potential modulators of CD45 PTPase. Heparin, ZnCl₂, spermine and NH₄-molybdate, in addition to suramin and vanadate, were able to inhibit PTPase function in these immunoprecipitates. Cyclosporine A (CsA), a strong

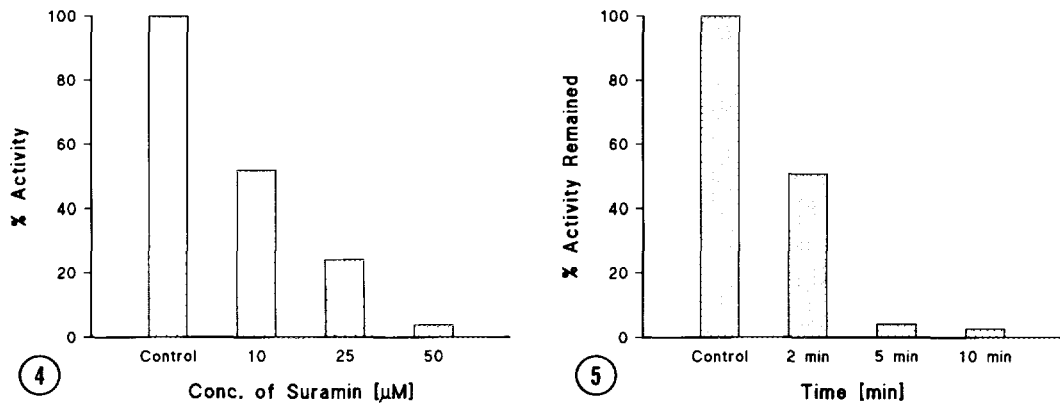


Fig. 4. Irreversibility of inactivation of CD45 PTPase by suramin. CD45 immunoprecipitates were incubated with 10, 25 and 50 μ M of suramin in PTPase buffer at 37°C in a total volume of 100 μ l for 10 min, then washed four times before assay using pNPP. A suramin-free control was run in parallel.

Fig. 5. Time course of inactivation of CD45 PTPase by suramin. Immunoprecipitates were incubated with 80 μ M suramin for the indicated times, washed four times in PTPase buffer, and then assayed for residual PTPase function using pNPP as substrate. The control samples were incubated for 10 min without suramin and then washed and assayed in parallel.

immunosuppressive agent, did not show appreciable inhibition of PTPase activity. Phenylarsine oxide, a known inhibitor of CD45 PTPase (32), is much less active than suramin in our experimental system.

DISCUSSION

Analysis of DNA sequences has revealed significant homology between different types of tyrosine phosphatases obtained from various sources (31, 33). Our results demonstrate that

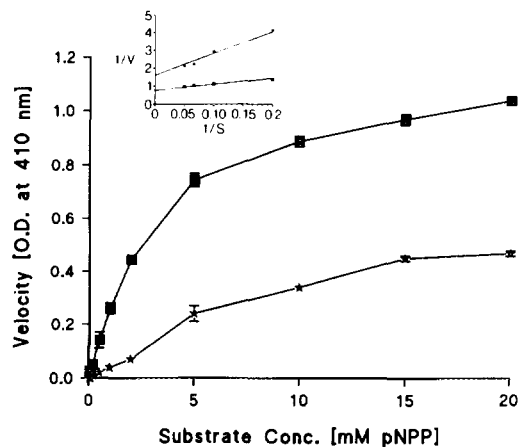


Fig. 6. Substrate/velocity curves for suramin-mediated inhibition of CD45 PTPase activity. PTPase was measured using various doses of pNPP in the absence (■) or presence (●) of 20 μ M suramin. Each point represents the mean of duplicate samples. A second experiment using an independently prepared lysate gave similar results. Inset shows Lineweaver-Burk plot of the same data showing non-competitive inhibition relative to the substrate (pNPP).

Table 1: Modulators of CD45-PTPase Activity

| Modulator | Amount | Activity (%) |
|----------------------------|----------|--------------|
| None (Control) | | 100 |
| ZnCl ₂ | 1 mM | 12.5 |
| MnCl ₂ | 1 mM | 62.7 |
| CaCl ₂ | 1 mM | 89.1 |
| MgCl ₂ | 1 mM | 109 |
| Heparin | 10 µg/ml | 52.8 |
| „ | 40 „ | 31.0 |
| „ | 200 „ | 7.5 |
| Spermine | 2 mM | 6.8 |
| Cyclosporine A | 50 µM | 100 |
| „ | 100 „ | 96.4 |
| Indomethacin | 100 „ | 87.0 |
| NH ₄ -Molybdate | 10 µM | 3.0 |
| „ | 40 „ | 0.0 |
| Na-K-Tartrate | 5 mM | 128 |
| Phenylarsine Oxide | 50 µM | 88.0 |
| „ | 100 „ | 77.4 |

PTPase activity of CD45 immunoprecipitates were measured in a total volume of 50 µl using pNPP as substrate. The immunoprecipitates were preincubated with the agents indicated for 10 min at room temperature with occasional gentle shaking, after which the reaction was started with the addition of 10 mM pNPP and incubated for 10 mins at 37°C.

suramin strongly inhibits T cell CD45 PTPase function in vitro, and suggest an explanation for the alteration of tyrosine specific phosphorylation of proteins observed in different cell types by this agent (24, 25). Suramin was shown to cause a dose-dependent reduction of maximal velocity which could not be overcome even at very high substrate concentration. Suramin similarly inhibited a variety of other protein tyrosine phosphatases isolated from diverse sources including rat brain (RPTP), yeast (YPTP) and vaccinia virus (VH1); (J. Ghosh., K. Guan and R. A. Miller: Unpublished observation). In other laboratories, suramin has been shown to inhibit a variety of other enzymes, including reverse transcriptase (15), protein kinase C (34), DNA polymerases (10), GTPase (35), and DNA topoisomerase II (36); except for reverse transcriptase, however, only partial inhibition is seen at clinically useful doses of suramin. Suramin-mediated inhibition of GTPase function in rat glioma cells was found to be noncompetitive (35), although both inhibition of PKC (34) and of MMLV-derived reverse transcriptase (15) were found to be attributable to competitive effects. In our experiments suramin-mediated inactivation of CD45 PTPase function was found to be rapid and essentially irreversible at drug concentrations well below those used clinically. Further work will be needed to determine the chemical alterations in the PTPase molecule that account for the loss of function.

Suramin exposure has previously been shown to increase the level of tyrosine-specific protein phosphorylation in the epidermal carcinoma cell lines KB and A431 (24) and several other prostate and colon cancer cell lines (25). Our work suggests that this increase may be due not to

an effect on tyrosine-specific kinases but rather to inhibition of one or more tyrosine-specific protein phosphatases. We hypothesize that the anti-proliferative, anti-viral, and immunosuppressive effects of suramin may also reflect its ability to alter the balance between kinase and phosphatase function in a variety of cell systems. Analysis of the antiphosphatase functions of suramin and its congeners may allow the design of new agents with improved therapeutic efficacies. Meanwhile, suramin may prove to be a useful reagent for the study of the activation and growth of various cell types whose reactions are controlled either directly or indirectly by tyrosine-specific phosphorylation.

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