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Sodium Stibogluconate Is a Potent Inhibitor of Protein Tyrosine Phosphatases and Augments Cytokine Responses in Hemopoietic Cell Lines

Manas K. Pathak and Taolin Yi

Using in vitro protein tyrosine phosphatase (PTPase) assays, we found that sodium stibogluconate, a drug used in treatment of leishmaniasis, is a potent inhibitor of PTPases Src homology PTPase 1 (SHP-1), SHP-2, and PTP1B but not the dual-specificity phosphatase mitogen-activated protein kinase phosphatase 1. Sodium stibogluconate inhibited 99% of SHP-1 activity at 10 μg/ml, a therapeutic concentration of the drug for leishmaniasis. Similar degrees of inhibition of SHP-2 and PTP1B required 100 μg/ml sodium stibogluconate, demonstrating differential sensitivities of PTPases to the inhibitor. The drug appeared to target the SHP-1 domain because it showed similar in vitro inhibition of SHP-1 and a mutant protein containing the SHP-1 PTPase domain alone. Moreover, it forms a stable complex with the PTPase: in vitro inhibition of SHP-1 by the drug was not removed by a washing process effective in relieving the inhibition of SHP-1 by the reversible inhibitor suramin. The inhibition of cellular PTPases by the drug was suggested by its rapid induction of tyrosine phosphorylation of cellular proteins in BaF3 cells and its augmentation of IL-3-induced Janus family kinase 2/Stat5 tyrosine phosphorylation and proliferation of BaF3 cells. The augmentation of the opposite effects of GM-CSF and IFN-α on TF-1 cell growth by the drug indicated its broad activities in the signaling of various cytokines. These data represent the first evidence that sodium stibogluconate inhibits PTPases and augments cytokine responses. Our results provide novel insights into the pharmacological effects of the drug and suggest potential new therapeutic applications. *The Journal of Immunology*, 2001, 167: 3391–3397.
effects of GM-CSF and IFN-α on TF-1 cell growth, suggesting broad activities of the drug in enhancing the signaling of various cytokines. These data provide novel insights into the pharmacological mechanism of sodium stibogluconate and suggest new therapeutic applications.

Materials and Methods

Chemicals and reagents

PTPase assay kits and GST fusion protein of PTP1B were purchased from Upstate Biotechnology (Lake Placid, NY). Suramin and potassium antimonyl tartrate was purchased from Sigma (St. Louis, MO). Sodium stibogluconate (its Sb content is 100 mg/ml and used to designate SS concentrations hereafter) was a gift from Dr. Xiaosu Hu (Sichuan Medical College, Sichuan, China). GST fusion proteins of SHP-1 (26) and SHP-2 (27) have been described previously and were prepared following established protocols (28). The GST fusion protein of SHP-1-ica was purified from DH5a bacteria transformed with a pGEX construct containing the coding region of the PTPase catalytic domain (aa 202–554) of murine SHP-1 (26), derived by PCR from the murine SHP-1 cDNA. The GST fusion protein of mitogen-activated protein kinase phosphatase 1 (MKP1) was purified from DH5a bacteria transformed with a pGEX construct containing the coding region of MKP1 cDNA derived by RT-PCR with the following primers (MKP1/5, 5′-ctggactcggctgtaagctggtgaag-3′; MKP1/3, 5′-aagtcgacgcagcttggggaggtggtgat). Murine IL-3 (29), recombinant human GM-CSF (30), and recombinant human IFN-α (31) have been described previously. Abs against phosphotyrosine (4G10; Upstate Biotechnology), β-actin (Amersham, Arlington Heights, IL), phosphotyrosine Stat5 (New England BioLab, Beverly, MA) and Janus family kinase 2 (Jak2; Affinity BioReagents, Golden, CO) were purchased from commercial sources.

In vitro PTPase assays

In vitro PTPase activities were measured using the commercial PTPase assay kit (Upstate Biotechnology) following established procedures (28). This assay measures the in vitro dephosphorylation of a synthetic phosphotyrosine peptide (R-R-I-E-D-A-E-pY-A-A-R-G). Brieﬂy, 0.01 μg GST/PTPase fusion proteins was incubated in 50 μl Tris buffer (10 mM Tris, pH 7.4) containing different concentrations of inhibitors or chemicals (0–1000 μg/ml) at 22°C for 10 min, followed by addition of 0.2 mM phosphotyrosine peptide and incubation at 22°C for 18 h; 100 μl Malachite Green solution were added and incubated for 5 min, and OD690 was measured after 5 min.

To assess the reversibility of inhibition of SHP-1 by PTPase inhibitors, GST/SHP-1 fusion proteins bound on glutathione beads were preincubated in cold Tris buffer or Tris buffer containing the PTPase inhibitors at 4°C for 10 min, followed by addition of 0.2 mM phosphotyrosine peptide and incubation at 22°C for 18 h; 100 μl Malachite Green solution were added and incubated for 5 min, and OD690 was measured after 5 min.

Results

Sodium stibogluconate inhibits protein tyrosine phosphatases in vitro

Through screening various chemical compounds by in vitro PTPase assays, we identified sodium stibogluconate as an inhibitor of PTPases. The dephosphorylation of a synthetic phosphotyrosine peptide by the GST/SHP-1 fusion protein was almost completely blocked (99%) by sodium stibogluconate at 10 μg/ml (Fig. 1A). Sodium stibogluconate also inhibited SHP-2 and PTP1B (Fig. 1A). However, ~10-fold higher concentrations of the drug (100 μg/ml) were required to achieve a similar degree (~99%) of inhibition of the two PTPases (Fig. 1A). Inhibition of SHP-1 by the known PTPase inhibitor suramin was less effective under comparable conditions (Fig. 1B). The drug showed no obvious inhibitory activity against MKP1 (34), a dual-specificity protein tyrosine phosphatase (Fig. 1C). Under the experimental conditions, the GST fusion proteins of SHP-1, SHP-2, PTP1B, and MKP1 showed similar PTPase activities against the peptide substrate (OD690 ~0.6 above background (0.03)) in the absence of inhibitors.

Sodium stibogluconate targets the SHP-1 PTPase catalytic domain and forms stable complexes with the phosphatase in vitro

Substrate dephosphorylation is mediated by the PTPase catalytic domain, the activity of which is often regulated by flanking N-terminal and C-terminal regions (5). To define whether sodium stibogluconate inhibits PTPases through targeting the PTPase catalytic domain or via the flanking regulatory regions, we compared the effect of sodium stibogluconate on the GST/SHP-1 fusion domain and forms stable complexes with the phosphatase in vitro.
protein and the GST/ShP-1cata fusion protein which contains the PTPase catalytic domain but has the Src homology 2 (SH2) domains and the C-terminal region deleted (Fig. 2A). Sodium stibogluconate showed similar activities in inhibiting the two proteins in their dephosphorylation of the phosphotyrosine peptide substrate in vitro (Fig. 2B), demonstrating that inhibition of SHP-1 PTPase activity by sodium stibogluconate does not require the SHP-1 SH2 domains and the C-terminal region. These results provide strong evidence that sodium stibogluconate directly targets the SHP-1 PTPase catalytic domain.

We next determined whether the in vitro inhibition of SHP-1 PTPase by sodium stibogluconate is a reversible process. For this, we examined whether washing the GST/ShP-1 fusion protein pre-incubated with sodium stibogluconate could relieve the inhibition. The inhibition of the GST/ShP-1 fusion protein by sodium stibogluconate was not affected by washing (Fig. 3). In contrast, the inhibition of the phosphatase by suramin was almost completely removed by the washing process (Fig. 3), consistent with a previous report (6).

**Sodium stibogluconate induces tyrosine phosphorylation of cellular proteins and augments IL-3-induced Jak2/Stat5 phosphorylation in Baf3 cells**

It is expected that the inhibition of PTPases in vivo will increase tyrosine phosphorylation of cellular protein substrates. To determine whether sodium stibogluconate functions as a PTPase inhibitor in vivo, we examined its effect on cellular protein tyrosine phosphorylation in the murine IL-3-dependent cell line Baf3. Treatment of Baf3 cells with sodium stibogluconate induced protein tyrosine phosphorylation (Fig. 4A) that was modest and transient in comparison with those induced by pervanadate (Fig. 4B). Increased tyrosine phosphorylation of cellular proteins of ~55 and 32 kDa was apparent in cells incubated with the drug for 5 min (Fig. 4, lanes 1-3). This induction of cellular protein tyrosine phosphorylation was dose dependent with more marked induction occurring at the higher drug concentration (Fig. 4, comparing lanes 2 and 3). Heightened phosphorylation of these proteins was also detected with prolonged treatment of 10, 30, or 60 min but at more modest levels (Fig. 4, lanes 4-12). This increased protein tyrosine phosphorylation was not due to variations in the protein samples as indicated by the similar amounts of β-actin protein in these samples (Fig. 4A, bottom). The drug showed no obvious effect on several other phosphotyrosine cellular proteins in the TCL samples (Fig. 4), suggesting certain specificity of the drug in induction of protein tyrosine phosphorylation. The identities of the 55- and 32-kDa proteins have not been determined. The weaker phosphorylation signal of p32 band in lane 1 of Fig. 4 compared with those of lanes 4, 7, and 10 was not consistently detected.

A functional role of SHP-1 in dephosphorylating the Jak family kinases during cytokine signaling has been documented (16, 18, 35-37). To determine whether sodium stibogluconate inhibits SHP-1 in vivo, we examined the effect of the drug on IL-3-induced Jak2 tyrosine phosphorylation in Baf3 cells (Fig. 5). Baf3 cells deprived of IL-3 were incubated with or without the drug for 10 min and then stimulated with IL-3 for various times. IL-3-induced tyrosine phosphorylation of Jak2 and Stat5 in Baf3 cells in the presence or absence of the drug. However, the phosphotyrosine levels of Jak2 and Stat5 in the presence of the drug were about twice those in cells without drug treatment as determined by densitometry analysis (Fig. 5; compare lanes 2–6 with lanes 8–12).

In cells unstimulated by IL-3, tyrosine phosphorylation of the two proteins was undetectable in the presence or absence of the drug (Fig. 5, lanes 1 and 7). Prolonged incubation with the drug...
myeloid leukemia cell line TF-1 responds to both GM-CSF, which promotes proliferation, and IFN-α, which inhibits cell growth. To determine whether the effect of the PTPase inhibitor is unique for the IL-3-initiated signaling events or affects other cytokines, we examined the growth responses of TF-1 cells to GM-CSF and IFN-α in the presence or absence of sodium stibogluconate.

Proliferation of TF-1 cells was induced by suboptimal concentrations of GM-CSF (5–40 ng/ml) in a dose-dependent manner (Fig. 7A). This proliferation of TF-1 cells was augmented in the presence of sodium stibogluconate at 50 μg/ml (Fig. 1A). No viable cells were detected in the cultures lacking GM-CSF in the presence or absence of the drug (Fig. 7A). These results demonstrated that sodium stibogluconate augmented the growth-promoting activity of GM-CSF in TF-1 cells but could not substitute the growth factor for maintaining cell viability or promoting growth under the experimental conditions.

In the presence of IFN-α, GM-CSF-induced proliferation of TF-1 cells was suppressed (Fig. 7B). Further reduction of GM-CSF-induced cell growth was detected in cultures containing both IFN-α and sodium stibogluconate (50 μg/ml; Fig. 7, B and C), indicating that the growth inhibition activity of IFN-α was enhanced in the presence of the drug. Because the enhanced growth inhibition of IFN-α by the drug occurred in the presence of GM-CSF, it indicated the dominance of the synergy between IFN-α and the drug over the activity of the drug in augmenting GM-CSF mitogenic signaling under the experimental conditions.

As shown in Fig. 7D, the activity of sodium stibogluconate in augmenting GM-CSF-induced TF-1 proliferation was dose dependent, with the optimal activity at 50 μg/ml. In contrast, more dramatic growth inhibition in the presence of IFN-α occurred at higher concentrations of the drug (Fig. 7E). Because the drug at low doses (12.5–50 μg/ml) showed no negative effect on GM-CSF-induced cell growth, its effect at such doses in augmenting IFN-α-induced growth inhibition was likely resulted from specific enhancement of IFN-α signaling. In contrast, nonspecific toxicity of drug at higher doses in combination with IFN-α might have contributed to the more dramatic growth inhibition.

The Sb(III) form of potassium antimonyl tartrate lacks inhibitory activity against PTPases

Sodium stibogluconate is of Sb(V) form which transforms inside cells into Sb(III) form that can affect Leishmania growth (38). We therefore determined the activity of potassium antimonyl tartrate of Sb(III) form in inhibiting PTPases in vitro and in vivo.

Unlike sodium stibogluconate, potassium antimonyl tartrate at 1–1000 μg/ml showed no detectable inhibition of PTPases SHP-1.
and PTP1B in vitro (Fig. 8A). It also failed to enhance IL-3-induced Stat5 phosphorylation (Fig. 8B) or IL-3-induced proliferation of Baα3 cells (Fig. 8C), indicating its lack of inhibitory activity against PTPases in vivo. Interestingly, it showed marked toxicity against Baα3 cells. The results together indicate that only the Sb(V) form acts as a PTPase inhibitor which is inactivated when transformed into the Sb(III) form.

Discussion

Sodium stibogluconate has been used clinically for decades in the treatment of leishmaniasis, caused by the protozoan *Leishmania*, which resides in macrophages (23). Although its pharmacological mechanism is poorly understood, there were indications that the therapeutic effect of this drug might be mediated via a cellular target(s); it kills intracellular *Leishmania* but has no effect on the free living form (promastigotes) that lives in the intestine of sand flies and can grow in defined culture medium in vitro (25).

Our data provide the first evidence that sodium stibogluconate is a potent inhibitor of PTPases in vitro and in vivo. Sodium stibogluconate inhibited the dephosphorylation of a synthetic phosphotyrosine peptide substrate by PTPases (SHP-1, SHP-2, and PTP1B) in vitro PTPase assays (Fig. 1). The dephosphorylation of p-nitrophenyl phosphate (Sigma) by these PTPases in vitro was also similarly inhibited by the drug (data not shown). The inhibitory activity of the drug against PTPases in vivo was indicated by the rapid induction of protein tyrosine phosphorylation of the two yet unidentified 56- and 32-kDa cellular proteins in Baα3 cells (Fig. 4). Interestingly, proteins of similar molecular mass had been found to be hyperphosphorylated in SHP-1-deficient cells in previous studies (29). Induced cellular protein tyrosine phosphorylation was less dramatic with prolonged drug incubation (Fig. 4), suggesting that the drug may be unstable under the experimental conditions or that the drug may sequentially inactivate PTPases with opposite effects on the phosphorylation of the cellular proteins. In this regard, it is interesting that PTPases were inhibited by the Sb(V) form of sodium stibogluconate which is known to transform in cells to the Sb(III) form that failed to show PTPase-inhibitory activity (Fig. 8). The intracellular transformation therefore could result in inactivation of the PTPase inhibitor and may

**FIGURE 8.** Potassium antimonyl tartrate lacks inhibitory activity against PTPases. A, Relative PTPase activities of GST fusion proteins of SHP-1, PTP1B, and MKP1 in the presence of various amounts of sodium stibogluconate (SS) or potassium antimonyl tartrate (PSbT). Data represent the mean values ± SD of triplicate samples measured by in vitro PTPase assays. B, TCL of Baα3 cells stimulated with IL-3 for various times in the absence or presence of sodium stibogluconate or potassium antimonyl tartrate was resolved in a SDS-PAGE gel, blotted to a membrane, and probed with Abs against phosphotyrosine Stat5 (pStat5) or the β-actin protein as indicated. The positions of phosphotyrosine Stat5 and β-actin are indicated on the right. C, Proliferation of Baα3 cells cultured in the presence of IL-3 (10 U/ml) and various amounts of sodium stibogluconate or potassium antimonyl tartrate for 3 days was measured by an MTT assay. Data represent the mean values ± SD of triplicate samples.
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account for the modest and transient induction of tyrosine phosphorylation by the drug and its modest effect on cell proliferation. This may have a beneficial side because it may be related to the lower toxicity of the drug in comparison to other PTpase inhibitors that allows its clinical application.

The inhibitory activity of sodium stibogluconate against PTpases in vivo was further indicated by the augmentation of IL-3-induced Jak2/Stat5 phosphorylation and IL-3-induced proliferation of BaF3 cells. We and others showed previously that SHP-1 dephosphorylates the Jak family kinases to down-regulate signaling initiated by cytokines (12, 16, 18, 35–37). Among the Jak kinases, IL-3 specifically activates the Jak2 kinase that phosphorylates the Stat5 protein to regulate gene expression (39). The observation that sodium stibogluconate augmented IL-3-induced Jak2/Stat5 tyrosine phosphorylation and IL-3-induced proliferation of BaF3 cells is therefore consistent with inhibition of SHP-1 by the drug in vivo. However, it remains possible that the effect of the drug on IL-3-induced Jak2/Stat5 phosphorylation and cell proliferation involves additional PTpases (e.g., the CD45 PTpase) that participate in dephosphorylating the Jak kinases (40). Indeed, sodium stibogluconate augmented G-CSF-induced Tyk2/Stat3 tyrosine phosphorylation in SHP-1-deficient cells (our unpublished data). The enhancement of IL-3-induced Jak2/Stat5 tyrosine phosphorylation by the drug was more substantial in later time points post-IL-3 stimulation, indicating induction of an extended period of phosphorylation by the drug. Such an effect of the drug suggests its targeting of PTpases recruited to Jak2/Stat5 at the later time points post-IL-3 stimulation to inactivate the signaling molecules.

Inhibition of PTpases in vivo by sodium stibogluconate was also consistent with the observation that the drug augmented the opposite effects of GM-CSF and IFN-α on TF-1 cell proliferation (Figs. 7 and 8). In particular, the observation suggested that the drug targeted PTpases that dephosphorylate shared signaling molecules (e.g., the Jak family kinases) used by both GM-CSF and IFN-α. Such a putative mechanism would explain the cytokine-dependent effects of the drug; its inhibition of PTpases leads to amplification of both mitogenic and growth inhibitory signals initiated by GM-CSF and IFN-α, respectively. It also suggests that drug may have broad activities in augmenting the signaling of various cytokines. SHP-1 has been shown in previous studies to down-regulate the signaling of GM-CSF (18) and IFN-α (12). It was reported (18) that macrophages from SHP-1-deficient mice show ~2-fold increase of GM-CSF-induced cell growth in comparison with controls. This level of growth increase is similar to the increase of GM-CSF-induced TF-1 cell growth in the presence of sodium stibogluconate, consistent with inhibition of SHP-1 by the drug. In light of the pathogenic effect of SHP-1-deficient monocytes/macrophages in the fatal motheaten phenotype (10), it is possible that the apparently modest effect of the drug on GM-CSF-induced cell growth could have significant biological consequences in vivo.

Our results also suggest that inhibition of PTpases by sodium stibogluconate at therapeutic concentrations to increase Jak/Stat phosphorylation and cellular responses to cytokines may be a major factor responsible for the pharmacological effect of the drug in the treatment of leishmaniasis. Among the cytokines that depend on Jak/Stat pathways for signal transduction (41), IFN-γ plays an important role in eliminating intracellular Leishmania (42). Moreover, impaired IFN-γ signaling was detected in Leishmania-infected macrophages and was associated with activation of SHP-1 by the parasite (43–46). Therefore, it could be postulated that sodium stibogluconate may augment IFN-γ signaling in macrophages via inhibiting SHP-1 (and other PTpases) and contribute to the clearance of intracellular Leishmania. Thus anti-Leishmania activity of sodium stibogluconate may derive both from augmenting cell signaling by Sb(V) and from parasite killing by Sb(III) transformed from Sb(V) inside cells. Further studies using Leishmania-infected macrophage cell lines will help to verify this hypothesis. Such a functional mechanism, nevertheless, is consistent with previous observations that modulation of host PTpases with specific inhibitors can effectively control the progression of Leishmania infection by enhancing cytokine signaling in macrophages (47–49). In light of the observation that anti-Leishmania drug sodium arsenite inhibits LPS-induced MAP kinase signaling in macrophages (50), modulation of cellular signaling could be a common mechanism of anti-Leishmania drugs.

The mechanism through which the drug inhibits PTpases is likely by targeting the PTpase catalytic domain of the enzymes. The drug was effective in inhibiting both the wild-type SHP-1 and the SHP-1 mutant containing the PTpase domain without the flanking N-terminal SH2 domains or the C-terminal region that regulate SHP-1 activity (Fig. 2). This mechanism is also consistent with the observation that the drug inhibited PTpB which, except for its PTpase catalytic domain, has no apparent structure similarity with SHP-1 and SHP-2 (34). In this regard, it is not unexpected that the drug showed no obvious activity against MKP1 because the amino acid sequence and structure of the catalytic domain of dual-specificity phosphatases are substantially different from those of the tyrosine-specific PTpases (34). Such a mechanism also suggests that the drug may have inhibitory activities against all tyrosine-specific PTpases that have the conserved PTpase catalytic domain. Although our results indicated that the drug formed a stable complex with SHP-1 in vitro that was resistant to a washing process, it is not clear at present whether this was due to docking of the drug into a pocket structure in the PTpase domain or involved the formation of covalent bonds. In the former case, it is likely that subtle differences in the putative pocket structure of PTpases may be responsible for the different sensitivities of the enzymes to the inhibitor in vitro. It also suggests the feasibility of developing chemical derivatives of the drug with more specific and potent activities against individual PTpases. Further studies to resolve the crystal structures of PTpases complexed with the drug will provide definitive answers in the future.

Demonstrated differential sensitivities of PTpases to the drug in vitro suggest similar differential sensitivities of PTpases in vivo, which may explain the dose-dependent effect of the drug on IL-3-induced cell proliferation and the known clinical side effect of the drug at higher dosages. Sodium stibogluconate augmented IL-3-induced BaF3 proliferation at therapeutic concentrations and suppressed cell growth at higher dosages. In clinical applications, sodium stibogluconate at therapeutic dosages was well tolerated by patients but is known at higher dosages to have side effects that include reversible nonspecific ECG changes and renal defects (25). Effects of the drug at higher dosages may be related to inhibition of PTpases that are sensitive to the drug only at higher concentrations.

Importantly, our finding that sodium stibogluconate was a potent inhibitor of PTpases and an enhancer of cytokine signaling suggests potential novel clinical applications for the drug in a variety of situations in which increased cytokine responses are beneficial. It is tempting to speculate coadministration of the drug with cytokines will improve the efficacy of existing cytokine therapies and reduce side effects and costs associated with cytokine therapies. Moreover, the drug by itself may have therapeutic effects through inhibiting PTpases to change the balance of intracellular tyrosine phosphorylation that controls cell proliferation, differentiation and...
functional activities. Suramin is presently being evaluated in clinical trials for the treatment of prostate cancer and other solid tumors (51). Because sodium stiboglucanate appeared to be a more efficient inhibitor of PTPases than suramin, it has the potential to become a better drug for effective treatment of these diseases.

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References