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Cancer cell resistance limits the efficacy of IFNs. In this study, we show that sodium stibogluconate (SSG) and IFN-α synergized to overcome IFN-α resistance in various human cancer cell lines in culture and eradicated IFN-α-refractory WM9 human melanoma tumors in nude mice with no obvious toxicity. SSG enhanced IFN-α-induced Stat1 tyrosine phosphorylation, inactivated intracellular SHP-1 and SHP-2 that negatively regulate IFN signaling, and induced cellular protein tyrosine phosphorylation in cancer cell lines. These effects are consistent with inactivation of phosphatases as the basis of SSG anticancer activity. Characterization of SSG by chromatography revealed that only selective compounds in SSG were effective protein tyrosine phosphatase inhibitors. These observations suggest the potential of SSG as a clinically usable protein tyrosine phosphatase inhibitor in cancer treatment and provide insights for developing phosphatase-targeted therapeutics. The Journal of Immunology, 2002, 169: 5978–5985.

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) are key signaling molecules and targets in developing novel therapeutics. The potential of such targeted therapeutics has been well demonstrated by the successful treatment of human chronic myelogenous leukemia and gastrointestinal stromal tumors with the PTK inhibitor STI-571 (1, 2), which targets bcrabl or c-kit aberrantly activated in the malignancies. Given the pivotal role of PTPases in intracellular signaling, inhibitors of the phosphatases might be expected to have therapeutic value. So far, few clinically usable inhibitors of PTPases have been reported despite extensive efforts in the last decade to identify them (3). Although a number of inorganic chemicals that broadly inhibit all PTPases are known (e.g., sodium orthovanadate, pervanadate, and iodoacetic acid), their usefulness as therapeutic agents has been limited due to their nonselective action and toxicity (3).

Our recent studies demonstrated that sodium stibogluconate (SSG), an anti-Leishmania drug with an unknown mechanism (4), is an inhibitor of selective PTPases in vitro and augments cytokine signaling and responses in hemopoietic cell lines (5). Among the PTPases sensitive to SSG inhibition, recombinant SHP-1 was inactivated by SSG at 10 μg/ml (5), a dosage within the clinically achievable levels of the drug in standard SSG therapy for leishmaniasis (20 mg/kg body weight) (6). Since SHP-1 is a negative regulator (7) in the Janus kinase (Jak)/Stat pathway that mediates the signaling of cytokines (8), SSG activity in augmenting cytokine signaling and responses in hemopoietic cells might be attributable to inactivation of the negative regulatory PTPase. This putative mechanism of SSG is consistent with the drug’s known anti-Leishmania action. SSG was shown to selectively kill Leishmania in host macrophages but not its free-living form (9), suggesting that the drug might act against the pathogen via targeting a host cellular molecule(s). Moreover, SSG anti-Leishmania activity was severely impaired in mice deficient of cytokines (e.g., IFN-γ, IL-4, or IL-12) (10–12), indicating that SSG functions via host cytokines. Given that the cytokines signal through the Jak/Stat pathway (8) and can activate macrophages to develop leishmanicidal activity (12, 13), SSG anti-Leishmania action might be mediated at least in part via inactivating negative regulatory PTPases to enhance the biologic effects of cytokines. This mode of action of SSG suggests that SSG might have potential novel applications.

IFNs are among the cytokines that signal through the Jak/Stat pathway (14) and have been approved for clinical usage for a number of diseases (15). For instance, IFN-α is beneficial in human malignancies, including melanoma (16). However, its clinical efficacy is often limited by resistance of cancer cells to the cytokine (16). Drugs that target IFN-α signaling molecules might augment IFN-α anticancer activity to overcome resistance but have not been reported so far. Given its putative mode of action, SSG might target negative regulatory PTPases to augment IFN-α signaling in cancer cells and enhance the activity of the cytokine against IFN-α-resistant cancer cells.

In this report, we demonstrate for the first time an anticancer activity of SSG that synergizes with IFN-α to eradicate IFN-α-resistant human cancer cells in vitro and in mice. We provide evidence that SSG functions as a PTPase inhibitor in cancer cells and augments IFN-α signaling. We further demonstrate that the PTPase inhibitory activity of SSG associates with selective compounds in the drug which is of heterogeneous formulation. As a clinically used drug with a novel mode of antitumor action and modest toxicity profile, SSG has the potential for incorporation into current anticancer therapies and might provide a basis for developing more effective and specific PTPase-targeted therapeutics.

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3 Abbreviations used in this paper: PTK, protein tyrosine kinase; SSG, sodium stibogluconate; PTPase, protein tyrosine phosphatase; Jak, Janus kinase; Sb, antimony; TCI, total cell lysate; CI, combination index.
Materials and Methods

Reagents, cell lines, cell culture, and cell growth inhibition assays

Human cancer cell lines DS (17), DR (17), WM9 (18), DU-145 (19), MDA-MB-231 (20), 5637 (21), U266 (22), SW620 (23), and SW480 (23) were obtained from colleagues at the Cleveland Clinic and cultured in RPMI 1640 medium supplemented with 10% FCS. Recombinant human IFN-γ (IFN-γ/H9251; England Biolabs), SHP-1 and SHP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased from commercial sources.

Animal studies

Athymic nude mice (nu/nu, NCR), 4 wk old (Tacofarm, Gent, NL), were inoculated (s.c.) in the flanks with WM9 human melanoma cells (3 × 10⁶ cells/site) on day 0. Starting on day 2, the mice were subjected to no treatment (control) or treatment with IFN-α (500,000 U, s.c., daily), SSG (12 mg, s.c., daily), or both (SSG/IFN-α). A separate group of nude mice with WM9 tumors were also treated with the SSG/IFN-α combination starting in the fourth week after cancer cell inoculation. The dose of IFN-α used for treatment was comparable to the dosage used in previous studies (5).

Detection of protein tyrosine phosphorylation.

For detection of Stat1 tyrosine phosphorylation, DR and WM9 cells were stimulated with IFN-α (50 U/ml) for various times and then treated with various amounts of SSG for 5 min. For detection of cellular protein tyrosine phosphorylation, cells were treated with various amounts of SSG for 5 min. Cells were lysed in cold lysis buffer (50 mM NaCl, 0.2 mM Na₂VO₄, 20 mM NaF, 1% Nonidet P-40, 2 mM PMSF, 0.1 M glycol, 1 mM sodium molybdate, 20% acetic acid). Total cell lysates (TCL) were separated in 10% SDS-PAGE gels, transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH), probed with specific Abs, and detected using an ECL kit (Amersham, Arlington Heights, IL).

Statistical analysis

Median effect analysis (26), which provides the most general form of studying the interactions between drugs, was used to analyze the interaction between SSG and IFN-α or IFN-β. Median effect plots were generated for IFNs alone, SSG alone, and the combinations in inhibiting the growth of WM9 cells in culture or WM9 tumors in nude mice. The combination index (CI) was determined and plotted vs fraction affected. Data were analyzed in both modes, mutually exclusive and mutually nonexclusive.

Results

SSG augments IFN-α-induced growth inhibition and Stat1 phosphorylation in IFN-α-resistant human lymphoma cell line DR in vitro

IFNs function via the Jak/Stat signaling pathway and have significant anticancer activity of IFNs and prompted us to determine whether SSG could overcome IFN resistance in human cancer cells. IFN-α-resistant DS lymphoma cell line (17,18) was used for treatment with IFN-α (50 U/ml) for various times in the combination with SSG for 5 min. Cells were lysed in cold lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 2 mM PMSF, and 20 mM sodium molybdate) and 0.2 mM phosphotyrosine peptide) at 22°C for 5 min before measurement of A₅₆₀ to quantify the amounts of free phosphate cleaved by the PTases from the peptide substrate. Ten percent content of individual samples was analyzed by SDS-PAGE/Western blotting to quantify the relative amounts of the phosphatase proteins.

EMSAs

EMSAs were performed as described previously (27) using a duplex of SSG IRSF probes, which were end-labeled with [γ-32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. Cell extracts were prepared from WM9 cells treated with IFN-α (50 U/ml) for various times in the presence or absence of SSG (10 μg/ml) and incubated with the probes for 20 min on ice to allow complex formation. The complexes were separated in 5% Tris-borate-EDTA buffer at 200 V for 2 h in 6% nondenaturing polyacrylamide gels and were detected by autoradiography after drying the gels.

Discussion

The effect of SSG on IFN-α-induced growth inhibition and Stat1 phosphorylation was studied in the human DS lymphoma cell line (17) (Fig. 1A). Such an augmentation was most obvious at lower doses of SSG, which at 12.5 μg/ml in combination with IFN-α resulted in 80% growth inhibition whereas the agents individually induced 40% growth inhibition (Fig. 1B). An augmenting effect of SSG at higher doses (i.e., 25 μg/ml) was also detectable, which was partially masked by the growth inhibitory effect of SSG as a single agent, and resulted in near-complete or complete killing of DR cells (Fig. 1B).

The effect of SSG on IFN-α-induced tyrosine phosphorylation of Stat1 in DR cells was assessed as an indicator of whether SSG functioned as a PTase inhibitor to augment IFN-α signaling in cancer cells. IFN-α stimulation of DR cells induced Stat1 tyrosine phosphorylation that was elevated in the presence of SSG (Fig. 1B).
IFN-resistant DR cell line in the presence of various amounts of SSG and/or blotting with an anti-SHP-1 Ab (upper panel). The CI was determined and plotted vs fraction affected (fa). When the CI < 1, drugs are synergistic, when CI = 1, drugs are additive, and when CI > 1, drugs are antagonistic. C, Median effect analysis of IFN-β-SSG interactions against WM9 cells under comparable conditions.

SSG interacts synergistically with IFN-α and IFN-β against WM9 human melanoma cell line in vitro

To further assess whether the activity of SSG in overcoming IFN resistance was restricted to DR cells or to IFN-α alone, effects of SSG on IFN-α- or IFN-β-induced in vitro growth inhibition of WM9 cell line of human melanoma were determined. IFN-α induces ~15% clinical response in patients with melanoma (29). Drugs that augments IFN signaling to overcome IFN resistance might therefore improve response rates.

SSG augmented IFN-α-induced in vitro growth inhibition of WM9 cells (Fig. 2A), against which IFN-α was only partially effective (Fig. 2A). IFN-α (1000 U/ml) resulted in 60% growth inhibition of WM9 cells, indicating a resistance of the melanoma cells to the cytokine. IFN-α-induced growth inhibition of WM9 cells was increased in the presence of SSG in a dose-dependent manner and resulted in near-complete or complete cell killing at SSG dosages of 50–100 μg/ml whereas SSG as a single agent caused partial growth inhibition (25–68%) of the melanoma cells. Thus, combination treatment overcame IFN-α resistance in WM9 melanoma cells in vitro.

To evaluate the nature of the interaction between SSG and IFN-α more rigorously, data of IFN-α and SSG combinations at various concentrations of each on WM9 cell growth in vitro (Fig. 2B, upper panel) were generated. Median effect analysis (26) of the data demonstrated that the interaction of the two drugs was synergistic (CI < 1; Fig. 2B, lower panel), although the calculated CI values were close to that of an additive interaction (CI = 1) when the drugs were used at higher doses (100–200 μg/ml and U/ml). SSG also interacted with IFN-β in a synergistic manner in inhibiting the growth of WM9 cells (Fig. 2C) under similar experimental conditions, demonstrating that SSG was also effective in combination with IFN-β against IFN-resistant melanoma cells.
SSG in combination with IFN-α eradicates IFN-α-resistant WM9 melanoma tumors in nude mice

SSG activity to overcome IFN resistance in vivo was investigated next via assessing the effects of SSG as a single agent or in combination with IFN-α against WM9 tumor xenografts that had been allowed to grow for 2 days in nude mice.

WM9 cells inoculated s.c. in nude mice formed tumors that grew aggressively in the absence of treatment (Fig. 3A), consistent with a previous report (18). SSG or IFN-α as single agents partially suppressed growth of WM9 tumors in nude mice (∼80 and 60%, respectively) by day 25 (Fig. 3A). Combination treatment of SSG and IFN-α for 2 wk resulted in tumor regression (Fig. 3A). Histologic evaluation of the tumor inoculation sites of two mice from each treatment group on day 25 confirmed the absence of microscopic tumors in SSG/IFN-α-treated animals (Fig. 4D) and the partial tumor growth inhibition in mice treated with the drugs individually (Fig. 4, B and C). The remaining mice in the SSG/IFN-α treatment group were observed for an additional 8 wk without treatment and no recurrence of tumor at the inoculation site was observed. The differences of tumor volumes between the groups on day 25 were highly significant (t test: control vs SSG, IFN-α and SSG/IFN-α, p < 0.01; SSG vs IFN-α, p < 0.01; SSG vs SSG/IFN-α, p < 0.01). The interaction between SSG and IFN-α against the WM9 tumors was synergistic (CI < 1) based on median effect analysis performed on data from day 25. The treatments of SSG, IFN-α, or the combination were nontoxic to the mice, which all survived until the end of the study and maintained their body weights as illustrated by the comparable body weights of control and SSG/IFN-α-treated mice (Fig. 3B). Histology of major organs (heart, kidney, liver, lung, and spleen) of the mice in the treatment groups was unremarkable (data not shown). Two mice of the combination treatment group were kept alive for an additional 8 wk without treatment and showed no obvious abnormality during that period. These results demonstrated an in vivo antitumor activity of SSG that synergized with IFN-α to eradicate IFN-α-refractory WM9 tumors in nude mice with no obvious toxicity.

The SSG/IFN-α combination was further evaluated in nude mice with large WM9 tumors that had been established for 4 wk before treatment onset. It markedly reduced tumor volume in the mice (Fig. 3C) that survived the treatment course of 12 wk with steady body weight gains (data not shown) in contrast to mice with similar WM9 tumor burden that usually died within 1–2 wk without treatment (24). Histologic evaluation of two mice in this group at the end of treatment course revealed no obvious abnormality in their major organs (data not shown). The remaining mice in the group were observed for an additional 2 wk without treatment. The residue tumors in the mice resumed aggressive growth similar to those in the control mice in Fig. 3A during that period (data not shown). Thus, the SSG/IFN-α combination was effective in shrinking large established WM9 tumors and was well tolerated during the long-term treatment course. Whether IFN-α and SSG interacted synergistically against the well-established tumors (Fig. 3C) as they did against the smaller tumors (Fig. 3B) or the cancer cells in vitro (Fig. 2) was not determined.

SSG inactivates intracellular SHP-2 and augments IFN-α-induced Stat1 tyrosine phosphorylation in WM9 cells

Given the synergistic activity of the SSG/IFN-α combination against WM9 tumors in mouse models, the effects of SSG on IFN-α signaling, PTPase activity, and cellular protein tyrosine phosphorylation in WM9 cells were further investigated.

FIGURE 3. Effects of SSG and its combination with IFN-α against WM9 human melanoma tumors in nude mice. A, Tumor volumes in mice inoculated with WM9 cells 2 days before subjecting to no treatment (Control) or treatment with SSG, IFN-α, or both. Data represent the mean ± SEM (n = 8). B, Body weights (mean ± SEM) of nude mice in the control or SSG/IFN-α combination treatment group during the treatment course. C, Tumor volumes in mice inoculated with WM9 cells 4 wk before treatment with the SSG/IFN-α combination for 12 wk. Data represent the mean ± SEM (n = 8).

FIGURE 4. Histology of WM9 cell inoculation sites in nude mice subject to differential treatments. Sections of WM9 cell inoculation sites of nude mice untreated (A) or treated with IFN-α (B), SSG (C), or the IFN-α/SSG combination (D) on day 25 were stained with H&E. Positions of the WM9 tumors are indicated by the arrows.
A, TCL of WM9 cells stimulated by IFN-α for 5 h and then treated without or with SSG for 5 min were analyzed by SDS-PAGE/Western blotting with Abs as indicated. Positions of molecular size markers (kDa) are shown on the left. Two phosphotyrosine proteins affected by SSG treatment are indicated by the arrows. D, WM9 cells were stimulated with IFN-α in the presence or absence of SSG. Cell extracts were prepared at different poststimulation time points and incubated with radiolabeled ISG561/ISRE probes. The DNA-protein complexes were resolved on nondenaturing 6% acrylamide gels and detected by autoradiography.

FIGURE 5. Effects of SSG on IFN-α-induced Stat1 tyrosine phosphorylation, ISGF3-ISRE complex formation, intracellular SHP-2 activity, and cellular protein tyrosine phosphorylation in WM9 cells. A, TCL of WM9 cells stimulated by IFN-α for 5 h and then treated without or with SSG for 5 min were analyzed by SDS-PAGE/Western blotting with Abs as indicated. Western blotting with an anti-SHP-2 Ab (upper panel) and SHP-2 (30) play a negative role in IFN signaling, although SHP-1 (28) and SHP-2 (30) play a negative role in IFN signaling, although SHP-1 expression is restricted to and thus functions mainly in hemopoietic cells (31). Consistent with its ubiquitous expression (32), SHP-2 protein was detected in WM9 cells (Fig. 5A). SHP-2 from WM9 cells treated with SSG showed reduced activities in comparison to that from untreated cells (Fig. 5B), demonstrating that SSG treatment resulted in partial inhibition of intracellular SHP-2 in WM9 cells. This result is consistent with our previous observation that recombinant SHP-2 could be inhibited by SSG in vitro but was less sensitive to the drug than SHP-1 (5). Given the negative regulatory role of SHP-2 in IFN signaling, inhibition of this PTPase might be involved in SSG augmentation of IFN-α signaling in WM9 cells.

Interestingly, treatment of WM9 cells with SSG as a single agent resulted in increased tyrosine phosphorylation in selective cellular proteins yet to be identified (Fig. 5C), providing additional evidence that SSG functioned as a PTPase inhibitor in WM9 cells. Since the drug by itself showed significant activity against WM9 cells in vitro (Fig. 2) and WM9 tumors in vivo (Fig. 3), these SSG-affected phosphotyrosine proteins could be involved in mediating the anticancer activity of SSG as a single agent.

SSG as a single agent or in combination with IFN-α inhibits in vitro growth of cell lines of other human malignancies

To further assess the potential of SSG as a novel anticancer drug, its effects on in vitro growth of cell lines of additional human malignancies were determined.

SSG as a single agent in a dose-dependent manner inhibited the growth of DU145 (prostate cancer), MDA231 (breast cancer), U266 (multiple myeloma), 5637 (bladder cancer), and SW620 and SW480 (colon cancer) cell lines in culture (Fig. 6). IFN-α (1000 U/ml) showed significant activity against DU145, MDA231, U266, and SW620 and SW480 cells and inhibited growth by 40–78% (Fig. 6). Higher doses of IFN-α (2,000–10,000 U/ml) as a single agent were not more effective when tested against DU145 cells in culture (data not shown). The growth inhibitory effect of IFN-α was augmented in the presence of SSG and resulted in complete cell killing at SSG dosages 25 μg/ml (MDA231), 50 μg/ml (SW620 and SW480), or 100 μg/ml (U266). In contrast, IFN-α showed only a minor effect against 5637 cells, consistent with a previous report (21). The SSG/IFN-α combination resulted in growth inhibition comparable to that induced by SSG alone, indicating a lack of interaction between the two drugs in 5637 cells in which the IFN-α signaling pathway is known to be defective due to a lack of p48 that functions downstream of Jak/Stat molecules (21).

FIGURE 6. Effects of SSG alone and in combination with IFN-α on in vitro growth of cell lines of human malignancies. Percentage of in vitro growth inhibition of DU145 (A), MDA231 (B), U266 (C), 5637 (D), SW620 (E), and SW4800 (F) cells in the presence of various amounts of SSG and/or IFN-α as determined by MTT assays. Data represent the mean ± SD values of triplicate samples.
These results further demonstrated an activity of SSG to interact with IFN-α in cells of various human malignancies. The observation that such an interaction was not apparent in 5637 cells indicated a requirement of the intact IFN-α signaling pathway for SSG-IFN-α interaction against cancer cells.

**PTPase inhibitory activity associates with selective compounds in SSG**

SSG consisted of pentavalent Sb in differential complex formation with gluconic acid (33). Given that Sb could covalently modify sulfhydryl groups (33) and that the sulfhydryl group of a cysteine residue conserved in all PTPases is required for PTPase activity (34), Sb in SSG might be responsible for the drug’s PTPase inhibitory activity. To address the issues whether selective or all compounds in SSG are effective PTPase inhibitors and whether PTPase inhibitory activity of SSG is solely defined by Sb, SSG was fractionated by chromatography. Sb content and PTPase inhibitory activity of individual fractions were determined.

Compounds in the SSG mixture were eluted in a time-dependent manner during chromatography, with most of them eluted between 8 and 25 min as revealed by mass spectrometry scanning (Fig. 7A). Consistent with a lack of compounds in fraction 1 (eluate of 0–8 min), no Sb was detected in the fraction by inductively coupled plasma mass spectrometry (Fig. 7A). Fractions 2–7 showed various amounts of Sb content with the highest levels detected in fractions 4 and 5 that accounted for 96% of total Sb in the eluates (Fig. 7A).

Inhibitory activities of the fractions and the parental SSG mixture against recombinant SHP-1 PTPase were assessed by in vitro PTPase assays. Consistent with our previous observation (5), SSG at Sb concentration of 10 μg/ml inactivated SHP-1 (Fig. 7B). As expected since it contained no detectable compounds or Sb (Fig. 6A), fraction 1 showed no activity against SHP-1 (Fig. 7B). Fractions 6 and 7 also failed to inhibit the PTPase although they had low levels of Sb (Fig. 7B). Interestingly, fraction 2, with an Sb level similar to those in fractions 6 and 7, was active against SHP-1 (Fig. 7B). In contrast, fractions 3 and 4 showed only minor effects on SHP-1 PTPase activity (Fig. 7B) despite the fact that their Sb levels were ~10- to 200-fold higher than that of fraction 2 (Fig. 7). Fraction 5 also showed a significant activity against SHP-1 although its Sb level was almost 100-fold higher than that of fraction 2 (Fig. 7B). Recombinant SHP-2 was also inhibited by fractions 2 and 5 but was not affected by the other fractions under comparable conditions (data not shown).

These results demonstrated that inhibitory activity against recombinant SHPs associated with selective compounds in the SSG mixture in a manner not solely defined by Sb contents. Since fraction 2 accounted for <10% of total compounds in SSG but was effective in inhibiting PTPases despite its relative low Sb concentration (Fig. 7A), it suggests that a small portion of the compounds in SSG is mainly responsible for the PTPase inhibitory activity of the drug.

**Discussion**

We showed in previous studies that SSG is an inhibitor of recombinant SHPs in vitro and augments the signaling and growth responses of hemopoietic cell lines to several cytokines (5). These observations suggested that SSG might have novel applications as a PTPase-targeted cytokine enhancer and prompted us to assess its potential to augment anticancer activity of IFN-α against human cancer cell lines resistant to the cytokine.

Our results herein demonstrate for the first time that SSG at a nontoxic dose has anticancer activity that synergizes with IFNs to overcome IFN resistance of human malignant cells in vitro and in mouse models. SSG interacted with IFN-α or IFN-β in vitro in a synergistic manner to prevent proliferation of IFN-resistant DR lymphoma cells (Fig. 1) and WM9 melanoma cells (Fig. 2). WM9 tumors in nude mice were eradicated following early treatment with the SSG/IFN-α combination for 2 wk, whereas the drugs individually induced only partial tumor growth inhibition (Fig. 3A). This combination treatment also induced rapid regression of large established WM9 tumors (Fig. 3C) and was well tolerated in a 12-wk treatment course, although it remains to be determined whether IFN-α and SSG interacted synergistically against the well-established tumors (Fig. 3C) as they did against the smaller tumors (Fig. 3B) or the cancer cells in vitro (Fig. 2). Melanomas are currently treated with IFN-α with moderate response rates (15–20%) and a median survival time of ~6–10 mo (29), which might be significantly improved by the addition of SSG. Moreover, our observation that SSG augmented IFN-α-induced growth inhibition of various human cancer cell lines suggests the potential of the SSG/IFN-α combination therapy for malignancies of different histology. Interestingly, the observation that SSG as a single agent at a nontoxic dose had activity superior to that of IFN-α against WM9 tumors provides the first evidence suggesting that SSG monotherapy might be beneficial in cancer treatment. In addition, the finding that SSG interacted synergistically with IFN-β against WM9 melanoma cells in culture indicates that SSG might be of value in IFN-β therapies which are currently used in the treatment of a number of diseases, including cancer (e.g., melanoma) (29) and multiple sclerosis (35). Moreover, the synergistic anticancer effects of the SSG/IFN combination (Figs. 2 and 3) and the ability of SSG to inhibit intracellular SHPs in malignant cells (Figs. 1 and 5) provided additional evidence supporting our hypothetic mode of action of SSG as a PTPase-targeted cytokine enhancer. It suggests potential synergistic effects of SSG in combination with other cytokines that might be exploited for therapeutic purposes.
Results of our studies also provide novel insights into the anticancer mechanisms of SSG, which might involve inactivation of different PTases in cancer cells and remain to be verified in future studies. The ability of SSG to synergize with IFNs might be mediated by inactivation of PTases negatively regulating the Jak/Stat pathway, resulting in augmentation of IFN signaling. SSG enhanced IFN-α-induced Stat1 phosphorylation that coincided with its inhibition of intracellular SHP-1 or SHP-2 in cancer cells (Figs. 1 and 4). Since both of the PTases are known to be negative regulators of IFN signaling (28, 30), their inhibition by SGG in cancer cells would result in augmentation of IFN-induced signaling and IFN anticancer activity. Such a mode of action is consistent with the observation that SSG, as a single agent, inhibited the growth of 5637 bladder cancer cells but failed to interact with IFN-α against these cells (Fig. 6D) in which the IFN-α signaling pathway has a defect downstream of the Jak/Stat molecules (21). In this regard, it is also interesting that only SHP-2 among the SHP pathway has a defect downstream of the Jak/Stat molecules (21).

In contrast, the anticancer activity of SSG as a single agent apparently functioned independently of IFN-α signaling and its negative regulatory PTases (e.g., the SHPs). This is indicated by the observations that SSG alone failed to induce Stat1 tyrosine phosphorylation (Fig. 1C) and that the drug as a single agent was capable of inducing growth inhibition in a cell line lacking an intact IFN-α signaling pathway (Fig. 6). The known functional roles of SHPs as negative signaling regulators also argue against their involvement in mediating SSG anticancer activity as a single agent. Since SHP-1 is also a key negative regulator of immunity (7), its inhibition by SGG might trigger enhanced immune responses. However, SGG anticancer activity as a single agent against WM9 melanoma cells is unlikely mediated via such a mechanism because it occurred in athymic nude mice (Fig. 3A) and was detectable in vitro (Fig. 2). On the other hand, the ability of SSG alone to increase cellular protein tyrosine phosphorylation in WM9 cells (Fig. 5C) could be of significance in this regard. It suggests that SSG might inactivate other PTases that mediate its anticancer activity as a single agent. Characterization of the SSG-inducible phosphotyrosine proteins may provide insights into the anticancer mechanism of SSG as a single agent and help to identify potential target PTase, which could be among the ones with oncogenic activity (37, 38). Although this putative mode of action of SSSG implicates more than one PTase as SSG targets in cancer cells, a limited PTase specificity of SSG in this case might be beneficial in that it allows the inhibition of different PTases to derive an anticancer effect. This is not without precedence because the inhibitory activity of STI-571 against both bcr-abl and c-kit PTKs was exploited for treating chronic myelogenous leukemia and gastrointestinal stromal tumor that each expresses one of the targeted PTKs (1, 2).

Our finding that SGG is a potent and clinically usable inhibitor of PTases with anticancer activity opens up potential new research areas for further mechanistic studies and for the development of more specific and effective PTase inhibitors as targeted therapeutics. SSG is a heterogeneous mixture of pentavalent Sb conjugated differentially to gluconic acid (33). The ability of Sb to form covalent bonds with the sulfhydryl group (33) and the existence of a conserved active site cysteine residue in catalytic pockets of all tyrosine phosphatases (34) suggest involvement of modification of the cysteine residue by pentavalent Sb in SSG as a potential inactivation mechanism. Since only selective compounds in SSSG were effective as PTase inhibitors that were not solely defined by Sb contents (Fig. 7), it suggests that only Sb conjugated with gluconic acid in certain specific configurations may gain access to the PTase catalytic pockets and allow optimal Sb/cysteine interaction, resulting in modification of the cysteine residue and PTase inactivation. Such an inhibitory mechanism could provide a rational explanation for the differential SSG sensitivities of PTases (5), each of which possesses a catalytic pocket of unique geometry for specific interaction with its substrates (34). It might therefore be feasible to develop more specific and effective inhibitors as phosphatase-targeted anticancer therapeutics through screening of SSG-related chemical compounds comprised of Sb conjugated to different organic moieties. Consistent with this hypothesis, glutamine (pentavalent Sb conjugated to methylglycine) was found to have PTase inhibitory activity that acted against a different spectrum of PTases compared with SSSG (our unpublished data). SSG may therefore represent a new class of PTase inhibitors that could be further developed as novel therapeutics and experimental tools. Moreover, the observation that PTase inhibitory activity associates with selective compounds in SSSG suggests the possibility that the active compounds in SSSG might be purified as a more potent and less toxic PTase-targeted therapeutic. It might also provide a basis to further define their chemical structures and interactions with targeted PTases for rational design of novel PTase inhibitors.

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References


