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# In Vitro Antileishmanial Properties of Tri- and Pentavalent Antimonial Preparations

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**To better understand the antileishmanial effects of antimonial agents we synthesized complexes of tri- and pentavalent antimony with mannan. The 50% inhibitory concentrations ($IC_{50}$s) of these agents, along with those of potassium antimony tartrate [Sb(III)] and sodium stibogluconate [Sb(V)], were determined for promastigotes and intramacrophage amastigotes. The trivalent antimonial agents were more potent than the pentavalent agents. Although the $IC_{50}$s were 60- to more-than-600-fold higher for promastigotes than for amastigotes, similar intracellular antimony concentrations in both life forms were measured after incubation with all four drugs at their respective $IC_{50}$s. Macrophages accumulated antimony during a 4-h exposure that was retained intracellularly for at least 3 days. Amastigotes inside macrophages had a higher antimony content 6 days after a single 4-h treatment than they did immediately after treatment, suggesting that macrophages serve as a reservoir for antimonial agents and prolong parasite exposure. Macrophages concentrated antimony from the medium with potassium antimony tartrate, trivalent antimony-mannan, and pentavalent antimony-mannan treatments. *N*-Acetylcysteine antagonized the antileishmanial effects of these three drugs against intracellular amastigotes; in contrast, it had minimal effects on the action of sodium stibogluconate.**

The leishmaniases are a group of diseases produced by invasion of the reticuloendothelial system of a vertebrate host by a parasite of the genus *Leishmania* followed by parasite multiplication inside macrophages. Symptomatic visceral leishmaniasis (kala-azar) is characteristically fatal if untreated. Antimonial chemotherapy increases the survival rate to over 90% (1). One of the first antimonial agents used was tartar emetic [potassium antimony(III) tartrate]. Unfortunately, this agent was highly toxic to humans as well as to *Leishmania* parasites. In the search for agents with higher therapeutic indices, a number of other antimonial agents were prepared and evaluated (for a review, see reference 24). This process produced the current drugs of choice, two closely related pentavalent antimony-carbohydrate complexes, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). Pentavalent antimony salts are 10-fold less toxic to mammals than trivalent salts (15). Likewise, these pentavalent antimony-containing drugs are less toxic to humans and have a higher therapeutic index than organic trivalent antimony preparations. Despite their clinical use for over half a century, the mechanism of action and basis for selective toxicity of these antileishmanial agents remain unknown.

Several properties of the pentavalent antimonial agents have been suggested to contribute to their activity. Carbohydrates form water-soluble complexes with antimony and may serve to deliver antimonial drugs to host macrophages. Relatively nontoxic pentavalent antimony may be a prodrug that is converted to more toxic trivalent antimony at or near the site of action. Interactions of antimony with key sulfhydryl groups may be a major mechanism of action and/or toxicity. The development of methods for measurement of intracellular antimony (22) along with an increased knowledge of the composition of sodium stibogluconate (23) and our synthesis of chemically analogous tri- and pentavalent antimony complexes have allowed further investigations of the significance of these hypotheses.

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## MATERIALS AND METHODS

Antimony trichloride (99%) and antimony pentachloride (95%) were obtained from Alfa/Johnson Matthey (Ward Hill, Mass.). Mannan from *Saccharomyces cerevisiae* and *N*-acetylcysteine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Potassium antimony tartrate (99.95%) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Sodium stibogluconate was obtained from the Centers for Disease Control (Atlanta, Ga.) and was extracted with diethyl ether to remove the *m*-chlorocresol preservative as previously described (23). All other chemicals were of at least reagent grade.

**Preparation of antimony-mannan complexes.** Mannan (100 mg) was dissolved in water (5 ml), cooled to 0°C, and treated with either antimony trichloride (275 mg) or antimony pentachloride (360 mg, 154 μl) with stirring (the molar ratio of antimony to anhydromannose was 2:1). After 15 min, the pH of the solution was adjusted to 7 by slowly adding 6 to 10 ml of 7.5% (wt/vol) aqueous sodium bicarbonate. The reaction mixture was heated at 60°C for 1 h, and after cooling to room temperature, insoluble material was removed by centrifugation (2,000 × *g* for 15 min). Methanol was added to the supernatant to a final concentration of 70%, and the precipitate that formed was isolated by centrifugation (1,000 × *g* for 15 min), dried in vacuo, and redissolved in 10 ml of water. The antimony concentrations of the solutions were measured by electrothermal atomization atomic absorption spectroscopy (22). The antimony recovered in methanol-precipitated Sb(III)-mannan averaged 1.1% ($n$ = 3; standard error [SE], 0.1%) of the initial antimony in the reaction mixture, and that in Sb(V)-mannan averaged 9.2% ($n$ = 2; SE, 0.7%). On a weight basis Sb(III)-mannan and Sb(V)-mannan contained 2 and 14% antimony, respectively.

**Promastigote growth inhibition.** Promastigote growth inhibition studies were performed on *Leishmania panamensis* WR120 (World Health Organization designation MHOM/PA/74/WR120) grown at 22°C in 5 ml of Schneider's drosophila medium containing 20% fetal bovine serum in 25-$cm^2$ tissue culture flasks as previously described (23). Promastigotes in log phase were seeded at a density of $1 \times 10^6$ to $2 \times 10^6$ per ml, drug treatment was initiated, and daily counts were made electronically with a model ZF Coulter Counter for 3 days. Growth rate constants were calculated from a plot of log parasite numbers versus time. The antimony concentration producing a 50% reduction in the growth constant ($IC_{50}$) was estimated from a least-squares linear regression of growth rate versus concentration.

**Intracellular amastigote growth inhibition.** Amastigote growth inhibition studies employed the J774G8 (hereafter referred to as J774) mouse macrophage cell line grown as previously described (23). Macrophages were grown to confluence at 34°C in 100 μl of complete RPMI medium per well in 96-well,

TABLE 1. $IC_{50}$s of antimony compounds for WR120 leishmanias in J774 macrophages[a]

| Compound | $IC_{50}$ for treatment | | | | | |
|---|---|---|---|---|---|---|
| | Continuous exposure (6 days) | 24-h pulse | 4-h pulse | 4-h pulse followed by *N*-acetylcysteine at: | | |
| | | | | 1 mM | 2 mM | 5 mM |
| Potassium antimony tartrate | 0.012 ± 0.002 (5) | 0.079 ± 0.016 (5) | 0.20 ± 0.02 (11) | 0.55 ± 0.11 (4) | 0.98 ± 0.15 (4) | 2.6 ± 0.4 (4) |
| Sb(III)-mannan | 0.038 ± 0.006 (6) | 0.10 ± 0.01 (3) | 0.29 ± 0.03 (5) | 0.67 ± 0.13 (3) | 0.87 ± 0.17 (3) | 1.7 ± 0.2 (3) |
| Sb(V)-mannan | 0.17 ± 0.03 (4) | 0.55 ± 0.07 (3) | 2.3 ± 0.4 (5) | 4.1 ± 1.3 (3) | 8.5 ± 0.9 (3) | 14.9 ± 0.5 (3) |
| Sodium stibogluconate | 12.7 ± 1.5 (6) | 50 ± 5 (5) | 98 ± 10 (13) | ND[b] | ND | 145 ± 12 (5) |

[a] Values are the means ± the SEs of *n* (shown in parentheses) independent determinations. The units used are micrograms of antimony per milliliter of medium.
[b] ND, not determined.

flat-bottom tissue culture plates. Suspensions of WR120 *L. panamensis* promastigotes grown in Schneider's medium (20% fetal bovine serum) in late log phase ($5 \times 10^7$ to $10 \times 10^7$/ml) were diluted with complete RPMI medium to a density of $2 \times 10^7$/ml. Aliquots (100 μl) containing $2 \times 10^6$ promastigotes were added to each well of the plate. After an overnight incubation, at the end of which all promastigotes were observed by inverted microscopy to have been phagocytosed, the old medium was aspirated. Antimonial preparations were serially diluted in complete RPMI medium, and aliquots (200 μl) were added in triplicate. In some experiments, the antimony-containing medium was replaced with fresh drug-free medium, sometimes containing other reagents as required, after 4 or 24 h. Alternatively, J774 cells were treated with antimony prior to infection with *Leishmania* promastigotes. After 4 h, the medium was replaced with fresh drug-free medium. At the designated times, promastigotes ($2 \times 10^6$ per well) were added.

After 5 days, leishmanial growth was assessed by the addition to each well of 1 μCi of [$^3$H]uracil (Moravek Biochemicals Inc., Brea, Calif.) in 50 μl of RPMI 1640. After overnight incubation, the parasites were harvested onto filter mats with a Skatron Combi Harvester (Skatron Inc., Sterling, Va.), the mats were sealed in plastic bags containing 10 ml of Beta Plate Scint cocktail (Wallac Inc., Gaithersburg, Md.), and radioactivity was counted in a model 1205 Betaplate counter (Wallac Inc.). Net counts per minute in drug-treated cultures (counts per minute in treated cultures minus counts per minute in uninfected controls) were expressed as a percentage of net counts per minute relative to infected, untreated controls. For each experiment, the concentration of antimony producing a 50% reduction of the net counts per minute ($IC_{50}$) was estimated from a log concentration plot.

**Promastigote binding and internalization assay.** A promastigote binding assay (17) was used to study the effects of antimony pretreatment of J774 macrophages. Late-log-phase promastigotes were incubated in Schneider's medium with [$^3$H]uracil (10 μCi/ml) overnight. After a washing, promastigotes ($2 \times 10^6$ per well) were added to J774 cells that had been previously treated with antimony and washed twice with fresh medium. Some wells were harvested immediately and counted as described above. A second set of wells was harvested and counted after 24 h. Inverted microscopy was used to verify that bound promastigotes had all been phagocytosed by this time.

**Amastigote isolation from J774 macrophages.** Macrophages were infected with promastigotes in 75-cm$^2$ culture flasks and 1 day later were treated with antimonial agents for 4 h. After two washings with antimony-free medium, cells were dissociated from the culture flask at the appropriate times by gentle scraping and ruptured by passage through a 0.5-in (1.27-cm) 27-gauge needle twice. The amastigotes were sequentially filtered through polycarbonate filters (Nuclepore, Pleasanton, Calif.) of 8-, 5-, and 3-μm pore sizes as previously described (12) before being centrifuged at 450 × *g* for 15 min. The supernatant was discarded, and the purified amastigotes were resuspended in RPMI medium (3 ml) for counting and antimony analysis.

**Estimation of macrophage volume.** The total volume of macrophages in aliquots of cells that had been treated with antimony for 4 h at the $IC_{50}$s of the various preparations was estimated by a modified quantitative buffy coat (QBC) procedure (25). First, QBC tubes (Becton Dickinson, Franklin Lakes, N.J.) were coated with acridine orange by pipetting 5 μl of 0.3% (wt/vol) acridine orange in methanol into each tube, inverting once to coat the walls, and drying at room temperature. A QBC float was inserted, and an aliquot (111 μl) of macrophages suspended in medium was aspirated. A small amount (~20 μl) of the gel from a Leukoprep tube (Becton Dickinson) was placed in the bottom endcap for the QBC tube with the end of a paper clip. The tube was then embedded in the gel in the endcap. After centrifugation for 5 min in a QBC centrifuge, the macrophages formed a layer midway up the float and resting on a layer of the gel. The height of the macrophage layer was measured with a QBC II manual counter, and the macrophage volume in microliters was estimated by multiplying the number of clicks by 0.002796.

**Quantification of intracellular antimony.** The intracellular antimony contents of promastigotes, amastigotes, and J774 macrophages were quantified by electrothermal atomic absorption spectroscopy by modification of a previously described method (22). Briefly, aliquots of cells were washed with antimony-free medium and centrifuged at 14,000 × *g* for 3 min. The supernatants were removed, and the tubes were inverted and drained at room temperature. The cells were digested with nitric acid (10 to 20 μl) overnight at room temperature and were diluted with water (90 to 180 μl) prior to antimony analysis. A Perkin-Elmer 4100 ZL atomic absorption spectrophotometer with Zeeman background correction and an autosampler were used. Sample aliquots (15 μl) were dried at 110°C for 20 s and 130°C for 30 s, charred at 800°C for 20 s, and atomized at 1,900°C for 5 s. The $A_{217.6}$ was read. Potassium antimony tartrate in dilute nitric acid (0.5%) was used for calibration.

## RESULTS

Mannan complexes with tri- and pentavalent antimony were prepared by a procedure previously described by Cantos and coworkers (6) for the synthesis of mannan-pentavalent antimony complexes. The reaction conditions employed were similar to those used to synthesize sodium stibogluconate, where equimolar amounts of aqueous stibonic and gluconic acids are reacted and neutralized with sodium hydroxide (10). In our syntheses, the mole ratio of antimony to anhydromannose in the reaction mixture was approximately 2:1 and antimony constituted 2 and 14% by weight of the Sb(III)- and Sb(V)-mannan products, respectively. For comparison, sodium stibogluconate is approximately 28% antimony by weight (16) and potassium antimony tartrate is 37.5% antimony.

The inhibitory effects of tri- and pentavalent antimony-mannan conjugates, potassium antimony tartrate [an Sb(III) derivative] and sodium stibogluconate on intracellular *Leishmania* amastigotes were investigated under a variety of exposure conditions. The results are summarized in Table 1. Potassium antimony tartrate was the most potent and sodium stibogluconate was the least potent of the four preparations tested. The antimony-mannan complexes were intermediate in potency, and the Sb(III)-mannan complex was more potent than the Sb(V)-mannan complex. For a given preparation, the $IC_{50}$ increased with decreasing exposure time. However, the increase in $IC_{50}$ was not proportional to the decrease in exposure time. After 4 h of exposure, the $IC_{50}$ was 2 to 4 times that for 24 h of exposure and 7 to 17 times that for continuous (6-day) exposure.

To investigate the role of sulfhydryl groups in antimony action, *N*-acetylcysteine was included with fresh drug-free medium added after intracellular amastigotes were exposed to antimony for 4 h. Treatment with *N*-acetylcysteine markedly increased the $IC_{50}$s of potassium antimony tartrate, Sb(III)-mannan, and Sb(V)-mannan in a dose-dependent fashion, while showing little effect on sodium stibogluconate activity (Table 1). While *N*-acetylcysteine alone did not affect the growth of intracellular amastigotes at concentrations of up to 5 mM, it directly inhibited promastigote growth ($IC_{50}$, 1.3 mmol/

TABLE 2. $IC_{50}$s and intracellular concentrations of antimony compounds[a]

| Compound | Results with promastigotes | | | Results with intracellular amastigotes | | | | |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ for continuous exposure[b] (μg of Sb/ml) | Intracellular antimony after 3 days | | $IC_{50}$ for 4-h exposure (μg of Sb/ml) | Intracellular antimony | | | |
| | | Content ($pg/10^6$ cells) | Concn[c] (μg/ml) | | Initial content ($pg/10^6$ cells) | Initial concn[d] (μg/ml) | Content after 6 days ($pg/10^6$ cells) | Concn[d] after 6 days (μg/ml) |
| Potassium antimony tartrate | 2.5 ± 0.3 (5) | 625 ± 43 (5) | 6.3 | 0.20 ± 0.02 (11) | 82 ± 6 (3) | 3.0 | 372 ± 20 (3) | 13.8 |
| Sb(III)-mannan | 2.4 ± 0.5 (3) | 588 ± 80 (5) | 5.9 | 0.29 ± 0.03 (5) | 78 (1) | 2.9 | 350 (1) | 13.0 |
| Sb(V)-mannan | 23.4 ± 5.5 (3) | 7,881 ± 1,223 (4) | 78.8 | 2.3 ± 0.4 (5) | 95 ± 8 (2) | 3.5 | 356 ± 28 (2) | 13.2 |
| Sodium stibogluconate | 8,700 ± 1,920 (6) | 5,653 ± 578 (4) | 56.5 | 98 ± 10 (13) | 894 ± 210 (3) | 33.0 | 1,869 ± 756 (3) | 69.2 |

[a] Values are the means ± the SEs of *n* (shown in parentheses) determinations.
[b] Promastigotes were continuously exposed to antimony for 3 days.
[c] Intracellular antimony concentrations were calculated for an estimated volume of 0.10 μl per $10^6$ promastigotes (3).
[d] Intracellular antimony concentrations were calculated for an estimated volume of 0.027 μl per $10^6$ amastigotes (3).

liter; SE, 0.11; $n = 2$), so we were unable to evaluate its effects on antimony action in promastigotes.

Ammonium chloride has previously been reported to prevent parasite destruction in the parasitophorous vacuole by Sb(V)-mannan, presumably because of an increase in intravacuolar pH (6). In our experiments, inclusion of 5 mM ammonium chloride, a concentration shown to increase the pH of the phagolysosome (20), with Sb(III)- and Sb(V)-mannan produced $IC_{50}$s of 0.034 μg (SE, 0.003; $n = 3$) and 0.18 μg (SE, 0.02; $n = 3$) of Sb per ml, respectively, for continuous exposure of amastigotes in J774 cells. These values are comparable to those observed for antimony-mannan complexes without ammonium chloride (Table 1).

We determined the continuous-exposure $IC_{50}$s of the four agents against promastigotes (Table 2). The $IC_{50}$s for the promastigotes ranged from 60- to more-than-600-fold higher than those for continuous exposure of amastigotes (Table 1). We also compared the $IC_{50}$s for promastigotes with the 4-h-exposure $IC_{50}$s for intracellular amastigotes (Table 2). The 4-h exposure conditions chosen for intracellular amastigotes simulate the duration of in vivo exposure dictated by the pharmacokinetics of sodium stibogluconate during standard once daily therapy, while continuous exposure of promastigotes was required to achieve toxicity at concentrations attainable without severely altering the composition of the growth medium. Potassium antimony tartrate and Sb(III)-mannan were the most potent, followed by Sb(V)-mannan and finally sodium stibogluconate, which was the least potent. Despite the much shorter exposure for intracellular amastigotes, the first three agents were 10-fold more potent against them than against promastigotes, while sodium stibogluconate was nearly 100-fold more potent. A comparison of promastigote intracellular antimony concentrations at the $IC_{50}$ for each of the four preparations revealed similar intracellular concentrations for both of the trivalent antimonial agents, and concentrations about 10-fold higher were observed for both of the pentavalent antimonial agents (Table 2).

The intracellular antimony concentrations in amastigotes were also determined after 4-h exposures at the $IC_{50}$ (Table 2). With all four drugs, intracellular antimony was detectable immediately after treatment and increased two- to five-fold after 6 days despite the absence of antimony in the medium. At both time points, potassium antimony tartrate, Sb(III)-mannan, and Sb(V)-mannan treatments produced similar antimony concentrations in amastigotes while sodium stibogluconate treatment generated much higher concentrations than the other agents.

To determine whether antimonial agents exhibit a postantibiotic effect, J774 macrophages were exposed for 4 h to various concentrations of potassium antimony tartrate or sodium stibogluconate prior to the addition of promastigotes at 0, 24, 48, and 72 h thereafter. The control $IC_{50}$s for potassium antimony tartrate and sodium stibogluconate were estimated in parallel by infection of J774 cells with promastigotes 1 day prior to the 4-h antimony treatment. The measured $IC_{50}$s were 56 to 65% of the control value for potassium antimony tartrate and 53 to 69% of the control value for sodium stibogluconate (Table 3). These reductions in $IC_{50}$s may be due to enhanced killing or decreased infectivity. To investigate the latter possi-

TABLE 3. Effects of WR120 promastigote addition time on the relative $IC_{50}$s for a 4-h exposure of J774 macrophages to potassium antimony tartrate and sodium stibogluconate[a]

| Promastigote addition time | Relative $IC_{50}$ (%) | |
|---|---|---|
| | Potassium antimony tartrate | Sodium stibogluconate |
| 24 h prior to antimony treatment (control) | 100[b] | 100[c] |
| Immediately after antimony treatment | 65 ± 10 ($n = 3$; $P = 0.06$[d]) | 69 ± 5 ($n = 4$; $P = 0.02$) |
| 24 h after antimony treatment | 56 ± 3 ($n = 3$; $P = 0.02$) | 56 ± 6 ($n = 3$; $P = 0.10$) |
| 48 h after antimony treatment | 62 ± 8 ($n = 2$; $P = 0.08$) | 53 ± 15 ($n = 2$; $P = 0.34$) |
| 72 h after antimony treatment | 65 ($n = 1$) | 41 ($n = 1$) |

[a] The relative $IC_{50}$s are expressed as a percentage of the control values and are the means ± the SEs of *n* (shown in parentheses) independent determinations.
[b] The mean $IC_{50}$ for these control experiments was 0.15 μg of Sb per ml.
[c] The mean $IC_{50}$ for these control experiments was 90 μg of Sb per ml.
[d] The paired *t* test (two tailed) was used to determine the statistical significance of differences between treatment and control groups.

TABLE 4. Antimony accumulation by J774 macrophages

| Antimonial treatment[a] | Antimony concn (μg/ml) | | |
|---|---|---|---|
| | In medium | Initial intracellular[b] | Intracellular/extracellular ratio |
| Potassium antimony tartrate | 0.20 | 8.6 ± 0.9 (5) | 43 |
| Sb(III)-mannan | 0.29 | 7.0 ± 0.9 (5) | 24 |
| Sb(V)-mannan | 2.3 | 8.8 ± 1.0 (5) | 3.8 |
| Sodium stibogluconate | 98 | 32.1 ± 5.7 (4) | 0.33 |

[a] J774 macrophages were exposed to the antimony compound at the indicated concentration (its $IC_{50}$) for 4 h. Then, the antimony-containing medium was removed, the cells were rinsed once with fresh medium, and the volume and antimony content were measured as described in Materials and Methods.
[b] The units shown are micrograms of antimony per milliliter of macrophage volume, and values are the means ± the SEs of *n* (shown in parentheses) determinations.

bility, J774 cells were pretreated with potassium antimony tartrate and sodium stibogluconate at concentrations up to twice the $IC_{50}$, infected with [$^3$H]uracil-labeled promastigotes, washed, harvested, and counted. The amounts of radioactivity retained in untreated and treated wells were identical immediately and after 24 h, suggesting that no decrease in infectivity occurred (data not shown). Microscopic examination 1 day after infection showed that nearly all of the promastigotes not removed by washing had been internalized.

Intracellular antimony concentrations were measured in macrophages after a 4-h treatment at the $IC_{50}$ of each agent (Table 4). Potassium antimony tartrate and Sb(III)- and Sb(V)-mannan each produced antimony concentrations of 7 to 9 μg/ml immediately after treatment. This result represented a 3.8- to 43-fold increase over the antimony concentration in the extracellular medium. Sodium stibogluconate treatment of macrophages at its $IC_{50}$ produced an intracellular antimony concentration of 32 μg/ml, a fourfold increase over those of the other three agents but only one-third of its concentration in the extracellular medium. Macrophages treated as outlined above with potassium antimony tartrate, Sb(III)-mannan, or Sb(V)-mannan and then incubated for 3 days in antimony-free medium retained all of the intracellular antimony initially present immediately after treatment, while sodium stibogluconate-treated macrophages retained 92% of the antimony initially present immediately after treatment. Antimony concentrations in amastigote-infected macrophages were identical to those observed in uninfected macrophages (data not shown).

## DISCUSSION

The possibility of metabolic conversion of pentavalent to trivalent antimony in vivo was suggested more than 50 years ago (13). Recently, hydride generation atomic absorption spectrometry analysis of serum and urine from patients treated with meglumine antimoniate revealed that 15 to 25% of serum antimony and 50% of urine antimony were trivalent (5, 21). Previous work in vitro with *Leishmania mexicana mexicana* promastigotes demonstrated that antimony sodium gluconate (Triostam), a trivalent analog of sodium stibogluconate, had a 50% lethal dose of 20 μg of Sb per ml, while sodium stibogluconate at 100 μg of Sb per ml had no effect (18). Potassium antimony(III) tartrate was shown to be substantially more potent than sodium stibogluconate against both promastigotes and amastigotes (23). These data are consistent with in vivo reduction of sodium stibogluconate to active trivalent antimony species.

Pentavalent antimony has been complexed with mannan, a polysaccharide recognized by macrophage mannose receptors, to target intracellular leishmanial infection (6). We have extended this work to compare the activities of tri- and pentavalent antimony complexed with mannan against both promastigotes and intracellular amastigotes in vitro. Complexation of $Sb^{3+}$ and $Sb^{5+}$ with the same carrier enabled direct comparison of their pharmacologic activities against *Leishmania* parasites. Trivalent antimony-mannan had an $IC_{50}$ comparable to that of potassium antimony tartrate against both promastigotes and amastigotes. Sb(V)-mannan was 10-fold less potent than Sb(III)-mannan but 100-fold more potent than sodium stibogluconate against both life stages.

Quantification of antimony concentrations in macrophages, amastigotes, and promastigotes was performed at the $IC_{50}$ of each drug for comparison after equipotent drug exposure. Antimony concentrations in macrophages were nearly identical after treatment with potassium antimony tartrate and Sb(III)- and Sb(V)-mannans as a result of differences in the extent of concentration (4- to 40-fold) from the extracellular medium. Amastigotes grown in macrophages treated with these three preparations had similar intracellular antimony concentrations, both immediately after a 4-h incubation in the drug and after 6 days of incubation in antimony-free medium. Additional antimony had accumulated after the 6-day incubation, presumably by macrophage-to-parasite transfer. After sodium stibogluconate treatment, the concentration of antimony in macrophages was higher than those for the other drugs but was only about one-third of the extracellular concentration. The concentration of antimony in amastigotes was nearly an order of magnitude higher than that achieved with the other three drugs, indicating that the antimony from sodium stibogluconate was intrinsically less toxic to the intracellular parasite.

When promastigotes were incubated at the $IC_{50}$ of each of the four preparations, the two trivalent preparations produced similar intracellular antimony concentrations that were an order of magnitude lower than concentrations achieved by the pentavalent preparations, indicating that pentavalent antimony is inherently less toxic to promastigotes. Comparison of intracellular with extracellular antimony concentrations revealed that potassium antimony tartrate, Sb(III)-mannan, and Sb(V)-mannan were concentrated intracellularly about threefold in promastigotes. Sodium stibogluconate was not concentrated, and the intracellular antimony concentrations were quite similar in both amastigotes and promastigotes. This similarity occurred despite the nearly 3 order of magnitude difference in the $IC_{50}$s and indicates that diminished susceptibility of promastigotes relative to amastigotes is due mainly to decreased uptake by promastigotes (and to a much lesser extent to decreased intrinsic toxicity). The phenomenon of decreased drug uptake by free-living forms has previously been described for pentamidine in trypanosomes (9).

Ammonium chloride has previously been reported to antagonize the action of Sb(V)-mannan on intracellular amastigotes in mouse peritoneal macrophages (8). In contrast, we found that 5 mM ammonium chloride, a concentration shown to increase the pH of the phagolysosome (23), had no effect on

the activity of any of the antimony preparations on amastigotes in macrophages. Furthermore, all agents were active against promastigotes. These results suggest that the acidic pH of the phagolysosome is not necessary for the action of any of the four agents investigated.

A proposed mechanism of action of trivalent organic arsenical drugs in trypanosomiasis involves complexation of arsenic with certain critical parasite sulfhydryl groups, including those on trypanothione (11). Antimony lies directly below arsenic in the periodic table, and the two are chemically quite similar. Antimonial drugs were developed as less-toxic analogs of arsenical drugs. Pentavalent antimonial agents are also effective against trypanosomes, particularly when used with difluoromethylornithine, an agent that decreases the intracellular concentration of trypanothione (14). Previous work has shown stable complex formation between antimony and leishmanial proteins after treatment with radiolabeled sodium stibogluconate (4). Therefore, the antiprotozoal action of organic antimonial agents may involve antimony binding to sulfhydryl groups. We examined the effect of the sulfhydryl reagent *N*-acetylcysteine on the toxicity of the antimonial drugs. This agent antagonized the action of potassium antimony tartrate, Sb(III)-mannan, and Sb(V)-mannan against amastigotes in macrophages in a dose-dependent fashion, with 5 mM *N*-acetylcysteine increasing the $IC_{50}$s by an order of magnitude. However, *N*-acetylcysteine had a minimal effect on the action of sodium stibogluconate. Since *N*-acetylcysteine had an effect on Sb(V)-mannan comparable to that on the trivalent antimony drugs, the oxidation state of antimony does not appear to be the primary factor in the failure of *N*-acetylcysteine to antagonize sodium stibogluconate. This result suggests that these preparations may be subject to different intracellular metabolic pathways, either in the macrophage or in the parasite. Whether critical leishmanial sulfhydryl groups are involved in sodium stibogluconate action remains to be determined.

An interesting aspect of therapy with sodium stibogluconate is an apparent resistance to reinfection in previously treated mice. Resistance was observed in liver but not in spleen or bone marrow and lasted for at least 6 days after the cessation of antimony treatment (7). This effect may have been due to persistence of the drug. When we treated macrophages with potassium antimony tartrate or sodium stibogluconate prior to infection, the $IC_{50}$s of both drugs were reduced by nearly 50% compared with those for infection followed by antimony treatment. This effect was observed for up to 3 days, the longest pretreatment period examined, and was not associated with a decreased level of infection. The increased susceptibility of *Leishmania* parasites in pretreated macrophages could have resulted from an increased drug susceptibility in transforming promastigotes or from an increased drug uptake as a result of exposure during the early stages of infection.

Macrophages treated with antimony preparations for 4 h and then incubated in antimony-free medium for 3 days retained most of the antimony present immediately after treatment. Clinically, sodium stibogluconate is effective with once daily dosing even though it has a short half-life in plasma and antimony levels in plasma fall below the $IC_{50}$ for leishmania for a substantial portion of the dosing period. This postantibiotic effect may result from antimony retention by macrophages. In a hamster model of visceral leishmaniasis, a single large dose of sodium stibogluconate was more effective than an equal amount divided into 6 or 12 doses over 3 days (2). In a murine model, the effectiveness of a single large dose was equal to that of the same total dose divided into seven daily doses (19).

In summary, trivalent antimony was more active than pentavalent antimony against both promastigotes and amastigotes by virtue of its increased uptake and increased inherent toxicity to promastigotes. However, both pentavalent antimonial agents tested exhibited in vitro activity against both life forms. The intracellular antimony concentrations at the $IC_{50}$ of a given agent were not greatly different in amastigotes and promastigotes. Exposure of macrophages to potassium antimony tartrate, Sb(III)-mannan, and Sb(V)-mannan for 4 h led to antimony concentration from the extracellular medium. Most of the antimony taken up after a 4-h exposure to each of the four agents remained inside macrophages for at least 3 days. Moreover, 6 days after treatment amastigote antimony concentrations were higher than they were immediately after treatment, suggesting continued delivery of antimony to the parasites. These results suggest that measures of antimony concentration in serum or of extracellular antimony concentration underestimate the concentrations to which amastigotes within macrophages are actually exposed during clinical treatment with sodium stibogluconate. They further suggest that the duration of this exposure is prolonged and is more likely to be reflected in the terminal antimony half-life of 76 h than in the initial half-life of 2 h (8).

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