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# The In Vivo Susceptibility of *Leishmania donovani* to Sodium Stibogluconate Is Drug Specific and Can Be Reversed by Inhibiting Glutathione Biosynthesis

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**Resistance to pentavalent antimonial ($Sb^v$) agents such as sodium stibogluconate (SSG) is creating a major problem in the treatment of visceral leishmaniasis. In the present study the in vivo susceptibilities of *Leishmania donovani* strains, typed as SSG resistant (strain 200011) or SSG sensitive (strain 200016) on the basis of their responses to a single SSG dose of 300 mg of $Sb^v$/kg of body weight, to other antileishmanial drugs were determined. In addition, the role of glutathione in SSG resistance was investigated by determining the influence on SSG treatment of concomitant treatment with a nonionic surfactant vesicle formulation of buthionine sulfoximine (BSO), a specific inhibitor of the enzyme γ-glutamylcysteine synthetase which is involved in glutathione biosynthesis, and SSG, on the efficacy of SSG treatment. *L. donovani* strains that were SSG resistant (strain 200011) and SSG sensitive (strain 200016) were equally susceptible to in vivo treatment with miltefosine, paromomycin and amphotericin B (Fungizone and AmBisome) formulations. Combined treatment with SSG and vesicular BSO significantly increased the in vivo efficacy of SSG against both the 200011 and the 200016 *L. donovani* strains. However, joint treatment that included high SSG doses was unexpectedly associated with toxicity. Measurement of glutathione levels in the spleens and livers of treated mice showed that the ability of the combined therapy to inhibit glutathione levels was also dependent on the SSG dose used and that the combined treatment exhibited organ-dependent effects. The SSG resistance exhibited by the *L. donovani* strains was not associated with cross-resistance to other classes of compounds and could be reversed by treatment with an inhibitor of glutathione biosynthesis, indicating that clinical resistance to antimonial drugs should not affect the antileishmanial efficacies of alternative drugs. In addition, it should be possible to identify a treatment regimen that could reverse antimony resistance.**

Resistance to the main therapeutic drugs, pentavalent antimonial ($Sb^v$) compounds such as sodium stibogluconate (SSG), is creating a clinical dilemma in the treatment of visceral leishmaniasis (1), with primary resistance now occurring in 30 to 65% of cases in some parts of India (S. Sundar, T. Jha, C. Thakur, M. Mishra, and R. Buffels, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 177a, p. 23, 2000). Studies with in vitro models have demonstrated that drug resistance can be related to a reduction in drug influx or an increase in drug efflux, qualitative or quantitative changes in the drug target (8, 19), or a combination of these factors. Molecular studies have identified a number of genes which may be important in controlling drug resistance in *Leishmania*, in particular, those which code for ATP-binding cassette (ABC) proteins. For example, the ABC transporter PgpA has been shown to be involved in resistance to arsenite and antimonial drugs since its gene is frequently amplified in selected drug-resistant parasites (19). Sequencing studies suggest that PgpA is a member of the multidrug resistance protein family, which consists of ABC transporters whose substrates include organic anions and drugs conjugated to glutathione, glucuronate, or sulfate. This has led to considerable interest in the role of antioxidant thiol compounds in *Leishmania* infectivity and drug resistance. The tripeptide glutathione (γ-glutamylcysteinylglycine) is ubiquitous in aerobic organisms, but trypanosomatids such as *Leishmania* and *Trypanosoma* also contain trypanothione, formed by conjugation of glutathione with spermidine (9). There is evidence that upregulation of genes involved in glutathione and trypanothione biosynthesis may correlate with increased resistance to metals (14, 15), and it is thought that efflux of arsenite and antimony involves their conjugation to thiols (18, 27). In addition, in vitro studies have shown that buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, an enzyme involved in glutathione and trypanothione biosynthesis, can reverse resistance to trivalent antimony in *Leishmania* (13). The thiol antioxidants may also be important in direct protection against reactive oxygen species generated by activated macrophages (7).

We have recently characterized different strains of *L. donovani* isolated from patients in India in 2000 as $Sb^v$ susceptible or resistant on the basis of their response to single-dose treatment with SSG at a dose of 300 mg of $Sb^v$/kg of body weight (3). These strains have been maintained without drug pressure since isolation; therefore, the mechanism responsible for decreased susceptibility to SSG seems to be a stable genetic change in the parasite. In this study the susceptibilities of our SSG-resistant and -sensitive strains to other antileishmanial

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drugs were determined to ascertain whether the changes in sensitivity to SSG treatment were drug specific or conferred cross-resistance to other antileishmanial agents. Cross-resistance to unrelated drugs with different molecular targets would indicate that a common mechanism, e.g., a nonspecific drug efflux pump, was responsible for the resistance observed. It is possible to determine the importance of particular cellular targets in drug susceptibility by using specific inhibitors or inducers in the presence of the drug. Therefore, in this study the importance of thiols in resistance to SSG was determined by comparing the effects of treatment of mice with a vesicular formulation of BSO in the presence and absence of SSG. A nonionic surfactant vesicle (NIV) formulation of BSO was used, since previous studies (21) have shown that this approach increases the efficacies of a variety of antileishmanial drugs by directing more of the drug dose to infected tissues.

## MATERIALS AND METHODS

**Materials.** SSG was provided by Glaxo Wellcome Ltd. (Ware, United Kingdom). $Sb^v$ (31.7% [wt/wt]) was provided by Glaxo Wellcome Ltd.. Fungizone is the proprietary brand of an amphotericin B (AMB)-sodium deoxycholate micellar complex, while AmBisome is the proprietary brand of liposomal AMB. Both were purchased from Munro Wholesalers, East Kilbride, United Kingdom. The nonionic surfactant tetraethylene glycol mono-*n*-hexadecylether was purchased from Chesham Chemicals Ltd., Harrow, United Kingdom. Paromomycin sulfate, hexadecylphosphocholine (miltefosine), and BSO were obtained from Sigma-Aldrich (Poole, United Kingdom). Two batches of BSO were used in the studies: one was bought >1.5 years before use (batch 1), and one was purchased <1 month before use (batch 2). All other reagents were of analytical grade.

**Animals and parasites.** Age-matched BALB/c mice (weight, 20 to 25 g; male or female mice inbred in-house) were used in this study. Commercially obtained Golden Syrian hamsters (*Mesocricetus auratus*; Harlan Olac, Bicester, Oxon, United Kingdom) were used for maintenance of the *L. donovani* strains. *L. donovani* strains 200011 and 20016, which were clinically derived from patients in India and collected under the regulations of the Bihar University Ethical Committee (3), were used in this study. The mice were infected by intravenous injection (tail vein, no anesthetic) with $1 \times 10^7$ to $2 \times 10^7$ *L. donovani* amastigotes (2). The day of parasite administration to the mice was designated day 0 of the experiment. Animal experiments were carried out in accordance with United Kingdom Home Office regulations and under ethical clearance from the University of Strathclyde's Ethical Committee.

**Vesicle formulations.** A total of 300 μmol of vesicle constituents, consisting of a 3:3:1 molar ratio of mono-*n*-hexadecylether tetraethylene glycol, cholesterol, and dicetyl phosphate, was melted by heating at 130°C for 5 min. The molten mixture was cooled to 70°C and hydrated with 5 ml of preheated (70°C) phosphate-buffered saline (PBS) to form empty NIV or aqueous BSO to form BSO-NIV. The vesicular formulations were homogenized at 8,000 ± 100 rpm for 15 min at 70°C with a Silverson mixer (model L4R SU; Silverson Machines, Chesham, United Kingdom) fitted with a 5/8-in tubular work head. The vesicle suspensions were stored at room temperature until use.

**In vivo efficacies of drug formulations.** Groups of infected mice ($n = 4$ or 5 per treatment in each experiment) received the relevant treatment either orally or intravenously. Mice treated orally were given PBS (controls) or miltefosine (15 or 25 mg/kg) on days 7 to 11 as a single daily dose. This dose was chosen on the basis of in vivo studies carried out by Murray and Delph-Etienne (24). Mice treated intravenously were dosed on day 7 with one of the following: PBS (controls), paromomycin (20 mg/kg), SSG solution (300 mg of $Sb^v$/kg), AMB deoxycholate (Fungizone; 0.375 mg of AMB/kg), or liposomal AMB (AmBisome; 8 mg of AMB/kg). A dose of 20 mg of paromomycin/kg was chosen, since previous studies have shown that this dose causes 50% suppression of liver *L. donovani* parasite burdens (29). AmBisome and Fungizone were prepared in accordance with the manufacturer's instructions and were diluted prior to use with 5% dextrose to give a final AMB concentration of 1 mg/ml. Animals were killed at 7 days posttreatment; and parasite burdens in the spleens, livers, and bone marrow were determined microscopically by blindly counting the number of parasites per 1,000 host nuclei from randomly chosen slides. Organ weights were used to calculate the Leishman donovan units (LDU) for spleen and liver parasite burdens (2). In order to determine the importance of glutathione in SSG susceptibility, the animals were treated intravenously on day 7 postinfection with PBS (controls), free SSG (70.4 to 300 mg of $Sb^v$/kg), free BSO, or BSO-NIV mixed 1:1 with free SSG or water. The doses used in each experiment are indicated in the Results section. The experiments were performed at least twice to confirm the results, and the parasite burdens were determined on day 7 posttreatment.

**Extraction of glutathione-thiol from tissue samples for analysis.** Spleen and liver samples collected at the time of killing were frozen in liquid nitrogen and stored at −70°C until extraction for glutathione analysis (within 2 weeks). Tissue samples (approximately 0.1 g) were homogenized in 5 volumes of ice-cold HEPES buffer (50 mM HEPES [pH 8.0], 5 mM EDTA) and then centrifuged at $10,000 \times g$ for 5 min. The supernatant was processed differently for spectrophotometric and high-pressure liquid chromatography analyses.

**Analysis of thiols by high-pressure liquid chromatography.** A 100-μl aliquot of the tissue extract supernatant was mixed with an equal volume of 2 mM monobromobimane in ethanol and heated at 70°C for 5 min. A total of 300 μl of 25% trichloroacetic acid was added, and the sample was centrifuged at $10,000 \times g$ for 3 min. Ten microliters of the supernatant was injected onto an Ace Hichrom $C_{18}$ column, and a gradient of 0.25% (vol/vol) glacial acetic acid in water to 0.25% (vol/vol) glacial acetic acid in methanol was added at a flow rate of 0.5 ml/min over 20 min. Elution of bimane-derivatized compounds was monitored by fluorescence detection with excitation at 398 nm and emission at 480 nm. Glutathione was identified and quantitated by comparison with glutathione standards (50 μM to 1 mM).

**Spectrophotometric analysis of total glutathione.** A 100-μl aliquot of the tissue extract supernatant was mixed with an equal volume of 10% trichloroacetic acid and frozen-thawed once before centrifugation at $10,000 \times g$ for 3 min. The total glutathione concentration ([GSH] and [GSSG]) was determined on the basis of a modification of the DTNB-GSSG recycling method described by Griffiths (12). Liver samples were diluted 10-fold and spleen samples were diluted 2-fold prior to assay. The assay was carried out in 125 mM sodium phosphate buffer (pH 7.4) containing 8 mM EDTA, with 100 μl of 6 mM DTNB, 100 μl of 1.9 mM NADPH, and 25 μl of sample or glutathione standard (final volume, 1 ml). The reaction was started by the addition of 5 μl of glutathione reductase (340 U/ml) and was monitored by determination of the increase in the absorbance at 412 nm. The rate of the reaction was proportional to the combined concentration of glutathione and GSSG, as determined with glutathione standards (20 to 100 μM).

**Presentation and statistical analysis of data.** Parasite suppression (mean ± standard error of the mean [SEM] percent suppression) was determined for a particular site by comparing each experimental parasite burden with the relevant mean burden for the control by using the LDU per organ for the spleen and liver and the number of parasites per 1,000 host cell nuclei for the bone marrow. Parasite burdens were analyzed by a Students' unpaired *t* test on the $\log_{10}$-transformed parasite burden data.

## RESULTS

**Miltefosine.** Oral dosing with miltefosine at 15 mg/kg by use of a multidose treatment regimen caused a significant reduction in liver parasite burdens in mice infected with strain 200016 (mean ± SEM percent suppression, 65% ± 10%; $P < 0.01$) and a significant reduction in both splenic (47% ± 10%; $P < 0.05$) and liver (86% ± 7; $P < 0.01$) parasite burdens in mice infected with strain 200011 (Table 1, experiment 1). An increase in the drug dose to 25 mg/kg resulted in a similar significant reduction in parasite burdens in all three sites surveyed compared to the control values (mean ± SEM percent suppression of strain 200016, 98.8% ± 0.2% for the spleen, 99.5% ± 0.3% for the liver, and 48% ± 18% for the bone marrow; mean ± SEM percent suppression of strain 200011, 98.8% ± 0.5% for the spleen, 99.6% ± 0.2% for the liver, and 97.8% ± 11.4% for the bone marrow [$P < 0.0005$]; Table 1, experiment 2). Treatment with free SSG caused a significant ($P < 0.0005$) reduction (99.5% ± 0.3%) in liver parasite burdens in mice infected with strain 200016 ($Sb^v$ sensitive) compared to those in the controls. SSG treatment also caused a significant ($P < 0.05$) reduction (67% ± 12%) in splenic parasite burdens

TABLE 1. Effects of drug treatment on burdens of different *L. donovani* strains[a]

| Expt no., strain, and treatment (dose) | Mean ± SEM % parasite burden (mean ± SEM % suppression) | | |
|---|---|---|---|
| | Spleen | Liver | Bone marrow |
| Expt 1 | | | |
| 200016 | | | |
| Control | 36 ± 13 | 2,197 ± 256 | 329 ± 125 |
| SSG | 40 ± 14 (21 ± 13) | 47 ± 15[b] (98 ± 1) | 293 ± 75 (25 ± 9) |
| Miltefosine, oral (15 mg/kg) | 27 ± 6 (27 ± 15) | 778 ± 228[c] (65 ± 10) | 258 ± 27 (25 ± 11) |
| Paromomycin (20 mg/kg) | 51 ± 2 (0 ± 0) | 1,420 ± 216[d] (35 ± 10) | 394 ± 103 (12 ± 12) |
| AMB deoxycholate (0.375 mg/kg) | 13 ± 2[c] (65 ± 4) | 74 ± 16[b] (97 ± 1) | 243 ± 33 (26 ± 10) |
| 200011 | | | |
| Control | 31 ± 7 | 2,250 ± 316 | 223 ± 16 |
| SSG | 32 ± 7 (20 ± 14) | 2,338 ± 475 (14 ± 9) | 247 ± 24 (3 ± 3) |
| Miltefosine, oral (15 mg/kg) | 17 ± 3 (47 ± 10[d]) | 310 ± 162[c] (86 ± 7) | 184 ± 40 (44 ± 12) |
| Paromomycin (20 mg/kg) | 37 ± 7 (8 ± 8) | 1,060 ± 208[c] (53 ± 9) | 307 ± 86 (25 ± 16) |
| AMB deoxycholate (0.375 mg/kg) | 13 ± 6[d] (59 ± 19) | 99 ± 25[b] (96 ± 1) | 99 ± 24[c] (70 ± 7) |
| Expt 2 | | | |
| 200016 | | | |
| Control | 50 ± 8 | 837 ± 91 | 156 ± 25 |
| SSG | 17 ± 6[d] (67 ± 12) | 5 ± 3[b] (99.5 ± 0.29) | 81 ± 29 (48 ± 18) |
| Liposomal AMB (8 mg/kg) | 0.25 ± 0.25[b] (99.5 ± 0.5) | 0 ± 0[b] (100 ± 0) | 1 ± 1[b] (97.5 ± 1.5) |
| Miltefosine, oral (25 mg/kg) | 0.1 ± 0.1[b] (99.8 ± 0.2) | 4 ± 3[b] (99.5 ± 0.31) | 4 ± 2[b] (97.5 ± 1.5) |
| 200011 | | | |
| Control | 61 ± 11 | 1,767 ± 191 | 244 ± 32 |
| SSG | 94 ± 15 (2 ± 2) | 1,913 ± 296 (8 ± 50) | 298 ± 48 (4 ± 2) |
| Liposomal AMB (8 mg/kg) | 3 ± 2[b] (94.8 ± 2.5) | 10 ± 5[b] (99.3 ± 0.4) | 2 ± 2[b] (99 ± 0.6) |
| Miltefosine, oral (25 mg/kg) | 1 ± 0.2[b] (99.8 ± 0.2) | 7 ± 4[b] (99.6 ± 0.2) | 6 ± 3 (97.8 ± 1.4) |

[a] *L. donovani*-infected mice were treated orally with miltefosine (days 7 to 11, once per day) or intravenously with SSG solution (222 mg of $Sb^v$/kg), AMB deoxycholate, liposomal AMB, or PBS (controls). Parasite burdens were assessed on day 14 postinfection, and the mean percent suppression for each treatment was determined.
[b] $P < 0.0005$ compared to the results for the relevant control.
[c] $P < 0.01$ compared to the results for the relevant control.
[d] $P < 0.05$ compared to the results for the relevant control.

in one experiment (Table 1, experiment 2), but this was not confirmed in a repeat experiment (Table 1, experiment 1). In contrast, similar treatment of mice infected with strain 200011 ($Sb^v$ resistant) failed to have any significant effect on parasite numbers in any of the three sites (Table 1).

**Paromomycin.** A single intravenous treatment with paromomycin at 20 mg/kg significantly reduced the liver parasite burdens in mice infected with either *L. donovani* strain (35% ± 10% [$P < 0.05$] for strain 200016; 53% ± 9% [$P < 0.01$] for strain 200011) compared to those in the relevant controls. However, this dose failed to have any significant effect on splenic or bone marrow parasite burdens compared to those in the controls (Table 1, experiment 1).

**AMB formulations.** Treatment with AMB deoxycholate (0.375 mg of AMB/kg) resulted in significant reductions in splenic (65% ± 4%; $P < 0.01$) and liver (97% ± 1%; $P < 0.0005$) parasite burdens in mice infected with *L. donovani* strain 200016 but had no significant effect on bone marrow parasite burdens compared to the burdens in the controls. Similar treatment of mice infected with *L. donovani* strain 200011 caused a significant reduction in parasite burdens in all three sites surveyed compared to the burdens in the controls (spleen, 59% ± 19% [$P < 0.05$]; liver, 96% ± 1% [$P < 0.0005$]; bone marrow, 70% ± 7% [$P < 0.005$]) (Table 1, experiment 1). Treatment with liposomal AMB at 8 mg of AMB/kg had a similar suppressive effect on the splenic, liver, and bone marrow parasite burdens of mice infected with *L. donovani* strain 200011 or 200016 (mean ± SEM percent suppression for strain 200016, 99.5% ± 0.5% for the spleen, 100% ± 0% for the liver, and 97.5% ± 1.5% for the bone marrow; mean ± SEM percent suppression for strain 200011, 94.8% ± 2.5% for the spleen, 99.3% ± 0.4% for the liver, and 99% ± 0.6% for the bone marrow; Table 1, experiment 2).

**BSO-NIV therapy.** Treatment with BSO-NIV (BSO dose, 34 mg/kg) had a strain-dependent effect which was apparent only when newly purchased BSO was used to prepare the formulation. Thus, treatment with BSO-NIV prepared with "old" (BSO batch 1) or "new" BSO (BSO batch 2) had no significant effect on the parasite burdens of mice infected with *L. donovani* strain 200011 and had no adverse effect on animal health (Table 2). Treatment of mice infected with strain 200016 with BSO-NIV formulated with either batch of BSO also had no adverse effect on animal health and no significant effect on bone marrow parasite burdens (Table 2). However, treatment with BSO-NIV prepared with batch 1 of BSO caused a significant ($P < 0.05$) increase in splenic parasite burdens in one experiment (Table 2, BSO batch 1), but this result was not obtained in another experiment with the same batch of BSO or two subsequent experiments with BSO-NIV prepared with fresh BSO (Table 2, batch 2). Treatment with BSO-NIV had no significant effect on liver parasite burdens compared to the burdens in the controls in two experiments with BSO-NIV

TABLE 2. Effects of different treatments on parasite burdens of mice infected with *L. donovani* strain 200011 or 200016[a]

| BSO batch, strain, and treatment (dose) | Mean ± SEM % parasite burden (mean ± SEM % suppression) | | |
|---|---|---|---|
| | Spleen | Liver | Bone marrow |
| Batch 1 | | | |
| 200016 | | | |
| Control | 18 ± 5 | 855 ± 102 | 145 ± 28 |
| SSG (282 mg of $Sb^v$/kg) | 11 ± 2 (40 ± 14) | 15 ± 6[b] (98 ± 1) | 43 ± 15[c] (72 ± 9) |
| BSO-NIV | 38 ± 6[c] (0 ± 0) | 850 ± 64 (7 ± 4) | 287 ± 47 (0 ± 0) |
| BSO-NIV and SSG | 5 ± 1[c] (72 ± 7) | 11 ± 8[b] (99 ± 1.0) | 11 ± 4[b] (97 ± 1) |
| 200011 | | | |
| Control | 10 ± 2 | 567 ± 117 | 101 ± 5 |
| SSG (282 mg of $Sb^v$/kg) | 8 ± 2 (30 ± 15) | 476 ± 107 (22 ± 15) | 106 ± 9 (6 ± 6) |
| BSO-NIV | 14 ± 1 (0 ± 0) | 486 ± 1,267 (13 ± 9) | 75 ± 25 (35 ± 20) |
| BSO-NIV and SSG | 58 ± 17[b] (0 ± 0) | 23 ± 9[b] (96 ± 2) | 87 ± 20 (21 ± 16) |
| Batch 2 | | | |
| 200016 | | | |
| Control | 22 ± 4 | 1,681 ± 189 | 305 ± 40 |
| SSG (70.4 mg of $Sb^v$/kg) | 30 ± 4 (5 ± 2) | 395 ± 60[b] (77 ± 4) | 367 ± 43 (25 ± 20) |
| BSO-NIV | 34 ± 7 (7 ± 7) | 571 ± 44[b] (65 ± 2) | 239 ± 36 (22 ± 12) |
| BSO-NIV and SSG | 0.5 ± 0.5[b] (98 ± 2) | 2 ± 2[b] (99.9 ± 0.1) | 82 ± 31[b] (73 ± 10) |
| 200011 | | | |
| Control | 58 ± 17 | 2,321 ± 282 | 402 ± 137 |
| SSG (70.4 mg of $Sb^v$/kg) | 68 ± 15 (9 ± 11) | 3,066 ± 496 (4 ± 4) | 426 ± 80 (10 ± 7) |
| BSO-NIV | 53 ± 18 (28 ± 14) | 2,835 ± 207 (0 ± 0) | 459 ± 45 (3 ± 2) |
| BSO-NIV and SSG | 438 ± 37[b] (0 ± 0) | 154 ± 37[b] (93 ± 1) | 962 ± 173[b] (0 ± 0) |

[a] Mice (four mice per group) infected with *L. donovani* strain 200011 or 200016 were treated intravenously on day 7 postinfection with PBS (controls), free SSG (264 or 70.4 mg of $Sb^v$/kg), BSO-NIV alone (BSO at 34 mg/kg), or BSO-NIV and SSG (final BSO dose of 34 mg/kg and SSG with 70.4 mg of $Sb^v$/kg; 2× formulations were mixed 1:1 just prior to dosing). Parasite burdens were determined on day 14 postinfection. The burdens among each group of control mice were not significantly different. Two batches of BSO were used in the experiments: batch 1 was used >1.5 years after purchase, and batch 2 was used within 1 month of purchase.

[b] $P < 0.001$ compared to the results for the relevant control.

[c] $P < 0.05$ compared to the results for the relevant control.

prepared with batch 1 of BSO (Table 2). However, a significant ($P < 0.001$) reduction in liver parasite burdens was obtained in two subsequent experiments with batch 2 of BSO (>77% suppression in parasite burdens compared to those in the controls; Table 2). Unfortunately, a direct comparison by use of BSO-NIV formulated with both batches of BSO could not be made since the supply of batch 1 was exhausted. Total and reduced glutathione levels in the spleen were unaffected by BSO-NIV treatment in mice infected with either strain compared to the levels in the controls (Table 3). BSO-NIV treatment also had no significant effect on the levels of reduced glutathione in the livers of mice infected with either strain compared to the levels in the controls but caused a significant ($P < 0.05$) reduction in total glutathione levels in the livers of mice infected with strain 200011 but not in the livers of those infected with strain 200016 (Table 3).

**SSG versus BSO-NIV and SSG therapy.** As expected, treatment with SSG alone (282 or 70.4 mg of $Sb^v$/kg; Table 2) had no significant effect on parasite burdens compared to those in the controls in mice infected with strain 200011. However, SSG treatment caused a significant ($P < 0.001$) reduction (98% ± 1%) in liver parasite numbers in mice infected with strain 200016 compared to the numbers in the relevant controls (Table 2). SSG treatment (282 mg of $Sb^v$/kg) had no significant effect on liver glutathione levels in mice infected with either strain compared to the levels in the controls (Table 3). However, both total ($P < 0.01$) and reduced ($P < 0.01$) levels of glutathione were significantly increased by SSG treatment in mice infected with strain 200011, whereas similar treatment of mice infected with 200016 did not affect the glutathione levels compared to the levels in the controls (Table 3).

Joint treatment of strain 200016-infected mice with BSO-NIV and SSG (BSO, 34 mg/kg; SSG, 70 mg of $Sb^v$/kg) caused a significant reduction in parasite numbers in the spleens, livers, and bone marrow compared to the numbers in the controls. This effect ($P < 0.001$) was independent of the batch of BSO used to prepare the BSO-NIV formulation (in four of four separate experiments; Table 2). The reduction in liver parasite burdens was similar to that obtained by treatment with free SSG at 282 mg of $Sb^v$/kg (Table 2). Similar treatment of mice infected with strain 200011 also unexpectedly caused a significant ($P < 0.001$) reduction in liver parasite burdens compared to those in the controls (in five of five separate experiments; Table 2).

BSO-NIV and SSG treatment resulted in significantly higher ($P < 0.001$) splenic parasite burdens compared to those in the controls (in five of five separate experiments; Table 2). BSO-NIV and SSG treatment either had no significant effect on bone marrow parasite burdens (in one of five separate experiments; Table 2) or caused a significant ($P < 0.001$) increase in bone marrow parasite burdens (in four of five separate experiments; Table 2). These effects were independent of the batch of BSO used to prepare the BSO-NIV formulation. The SSG dose was reduced for joint treatment since preliminary studies

TABLE 3. Effects of different treatments on spleen and liver glutathione levels in *L. donovani*-infected mice[a]

| Glutathione type, strain, and treatment | Mean ± SEM glutathione level (mM) | |
|---|---|---|
| | Spleen | Liver |
| Reduced glutathione (HPLC assay) | | |
| 200016 | | |
| Control | 1.21 ± 0.25 | 3.02 ± 0.21 |
| BSO-NIV | 1.81 ± 0.15 | 3.10 ± 0.58 |
| BSO-NIV and SSG | 2.19 ± 0.47 | 4.04 ± 0.18[b] |
| SSG | 1.78 ± 0.25 | 4.36 ± 0.19[b] |
| 200011 | | |
| Control | 0.97 ± 0.28 | 3.28 ± 0.27 |
| BSO-NIV | 1.67 ± 0.11 | 3.06 ± 0.23 |
| BSO-NIV and SSG | 2.35 ± 0.23[b] | 4.23 ± 0.27[c] |
| SSG | 2.04 ± 0.05[b] | 3.11 ± 0.08 |
| Total glutathione (DTNB assay) | | |
| 200016 | | |
| Control | 2.50 ± 0.05 | 7.21 ± 0.42 |
| BSO-NIV | 2.75 ± 0.31 | 6.66 ± 0.53 |
| BSO-NIV and SSG | 3.65 ± 0.23[b] | 5.94 ± 0.70 |
| SSG | 2.43 ± 0.22 | 8.44 ± 0.29 |
| 200011 | | |
| Control | 2.50 ± 0.20 | 7.19 ± 0.41 |
| BSO-NIV | 2.40 ± 0.08 | 5.95 ± 0.25[c] |
| BSO-NIV and SSG | 3.42 ± 0.32[b] | 6.29 ± 0.60 |
| SSG | 3.10 ± 0.01[c] | 6.97 ± 0.17 |

[a] Spleen and liver glutathione levels in mice treated with BSO batch 1 (Table 2).
[b] $P < 0.01$ compared to the results for the relevant control.
[c] $P < 0.05$ compared to the results for the relevant control.

showed that the use of SSG at higher doses (>138 mg of $Sb^v$/kg) resulted in a significant suppression in parasite burdens in all three sites (at day 7 posttreatment in animals infected with strain 200011, the burdens were suppressed 82% in the spleen, 94% in the liver, and 94% in the bone marrow compared to those in the controls). However, it also resulted in significant acute toxicity, as demonstrated by a significant reduction in both body and spleen weights, and some of the animals had to be euthanized at 2 days posttreatment.

Joint treatment with BSO-NIV and SSG with SSG at a dose >138 mg of $Sb^v$/kg resulted in significant reductions in total and reduced glutathione levels in the liver compared to the levels in the controls (data not shown). Treatment with BSO-NIV and SSG, with SSG at 70 mg of $Sb^v$/kg, was associated with significant increases in the levels of reduced glutathione in the livers of infected mice (for strain 200011, $P < 0.05$; for strain 200016, $P < 0.01$; Table 3), whereas total glutathione levels were similar to the levels in the controls (Table 3). This treatment also caused a significant increase ($P < 0.01$) in total glutathione levels in the spleens of mice infected with either strain (Table 3) and a significant ($P < 0.01$) increase in reduced glutathione levels in the spleens of mice infected with strain 200011 (Table 3). Reduced glutathione levels were higher in the spleens of mice infected with strain 200016, but the results were not significantly different compared to those for the controls (Table 3). In a repeat experiment, treatment with BSO-NIV and SSG, with SSG at 70 mg of $Sb^v$/kg, had no significant effect on glutathione levels compared to the levels in the controls (data not shown). However, this treatment did cause a significant ($P < 0.04$) increase in the level of reduced glutathione in the livers of mice infected with strain 200011 compared to the levels in the controls.

Joint treatment with BSO-NIV and SSG was associated with a significant increase in spleen weights in mice infected with either strain (weights for mice infected with strain 200016, 0.18 ± 0.01 g for control mice and 0.32 ± 0.01g for mice treated with BSO-NIV and SSG; weights for mice infected with strain 200011, 0.19 ± 0.03 g for control mice and 0.50 ± 0.03 g for mice treated with BSO-NIV and SSG). This increase in spleen weight did not occur in mice treated with free SSG or BSO-NIV alone (data not shown).

## DISCUSSION

Resistance to antimonial drugs is becoming a major problem in the treatment of visceral leishmaniasis (4; Sundar et al., 40th ICAAC). Therefore, in areas with a high incidence of clinical nonresponsiveness, alternatives to $Sb^v$ which are affordable to the local medical services need to be used (23). The results of this study indicate that decreased susceptibility to SSG is drug specific and does not confer cross-resistance to paromomycin, miltefosine, or AMB. This lack of cross-resistance correlates with both experimental and clinical findings. Thus, antimony-resistant *L. infantum* axenic amastigotes, induced by in vitro drug pressure, were as susceptible to pentamidine and AMB as wild-type clones (26). AMB treatment resulted in long-term cure in >90% of the patients who did not respond to antimony treatment (23), and multiple doses of paromomycin (aminosidine) or miltefosine gave high cure rates in an area of India where antimony resistance is prevalent.

Studies have indicated that resistance to heavy metals such as antimony or arsenic in *Leishmania* is due to the ability of the parasite to limit drug exposure by forming metal-thiol conjugates with trypanothione, a parasite-specific thiol (5, 20). These conjugates are then sequestered into an intracellular vesicle before extrusion by specific transporters (15, 18, 19). The results of this study indicate that glutathione biosynthesis has a major role in SSG resistance since treatment with vesicular BSO, a specific inhibitor of γ-glutamylcysteine synthetase, significantly enhanced the therapeutic efficacy of SSG against both SSG-resistant and -susceptible strains of liver parasites. However, it is not possible to determine whether the effect involved just glutathione, since BSO inhibits both glutathione and trypanothione biosynthesis in trypanosomatids such as *Leishmania* and *Trypanosoma* (9). The reason for the ability of BSO-NIV and SSG treatment to significantly increase the splenic parasite burdens in mice infected with strain 200011 (an SSG-resistant strain) but decrease the parasite burdens of mice infected with strain 200016 (an SSG-susceptible strain) is under investigation.

Combined treatment with BSO-NIV and SSG was toxic at high SSG doses (>138 mg of $Sb^v$/kg). In vitro studies with metal-resistant cell lines have shown that depletion of glutathione after BSO treatment results in metal accumulation and reverses drug resistance (6, 16). Therefore, the toxicity may have been due to higher tissue antimony levels since antimony is known to be toxic and can cause side effects such as nausea,

vomiting, diarrhea, cardiotoxicity, hepatic damage, respiratory problems (30), and weight loss (25). Mice treated with SSG (>138 mg of $Sb^v$/kg) and BSO-NIV in this study did exhibit a high level of weight loss and some difficulty with their breathing. These effects were eliminated by reducing the dose of SSG used. Glutathione plays a central role in protecting against oxidative stress, but simply inhibiting this function was not responsible for the toxicity observed, since treatment with the same dose of BSO-NIV alone did not result in any toxic side effects. The toxicity associated with a single-dose BSO-NIV and SSG treatment perhaps indicates that antimony had accumulated in tissues as the $Sb^{III}$ form, which is much more toxic to the host and parasite (27, 30). Incubation of the pentavalent antimonial drug, meglumine antimoniate, with glutathione results in its reduction to the trivalent form (11), perhaps indicating that bioreduction may occur before or upon drug conjugation to glutathione.

The anticipated reductions in glutathione levels and treatment efficacy in mice treated with BSO-NIV and SSG were not obtained. For example, treatment caused a significant reduction in splenic parasite burdens compared to those in the controls in mice infected with SSG-susceptible strain 200016 and a significant increase in parasite burdens compared to those in the controls in mice infected with SSG-resistant strain 200011 but had no effect or caused an increase in spleen glutathione levels on day 7 posttreatment. The effect of SSG and BSO-NIV treatment on glutathione levels depended on the dose of SSG given, since the BSO-NIV dose remained constant. Thus, the use of SSG at high doses (>138 mg of $Sb^v$/kg) with BSO-NIV treatment resulted in significant reductions in total and reduced glutathione levels in the liver and spleen, whereas the use of SSG at a low dose (70.4 mg of $Sb^v$/kg) caused a significant increase in the levels of reduced glutathione in the livers of infected mice, irrespective of the parasite's susceptibility to SSG, and had no effect on total glutathione levels. Thus, the effect of modifying intracellular glutathione levels depended on the concomitant level of oxidative stress present ($Sb^v$ levels). Longitudinal studies are required to understand the role of glutathione in the outcome of SSG treatment, since levels in tissues were determined only on day 7 posttreatment in this study.

Studies are also under way to quantify tissue antimony ($Sb^v$ and $Sb^{III}$) levels to ascertain whether these are affected by the treatments. It may be possible to identify a more effective combination therapy which can reverse SSG resistance without inducing significant toxicity. This could be achieved by reducing the dose of BSO-NIV given, altering the interval between BSO-NIV and SSG dosing, or using an NIV formulation of DL-α-difluoromethylornithine, a specific irreversible inhibitor of ornithine decarboxylase (10) which would inhibit trypanothione production but not affect glutathione synthesis. However, this approach may be unsuccessful in *Leishmania*, since the drug may be poorly taken up by the parasite or may have a low affinity for its target proteins or salvage pathways may overcome its inhibitory effect (22).

In this study the effect of vesicular BSO treatment depended on the strain used (an SSG-resistant or -sensitive strain) and the age of the BSO used. Only treatment of mice infected with SSG-sensitive strain 200016 with BSO-NIV prepared from freshly purchased BSO had a consistent significant suppressive effect on liver parasite burdens. This result perhaps indicates a stability problem with BSO, although it was stored as recommended by the manufacturer. BSO has already been shown to have antileishmanial properties, since in vitro treatment of *L. donovani*-infected macrophages with 5 mM BSO reduced the percentage of cells infected and the mean number of parasites per cell (17).

In summary, this study showed that the resistance to SSG exhibited by clinically derived strains of *L. donovani* was not associated with cross-resistance to other classes of compounds and could be reversed by treatment with an inhibitor of glutathione biosynthesis. In addition, it indicates that the high incidence of clinical resistance to antimonial drugs should not affect the clinical response to alternative drugs and that it should be possible to identify a treatment regimen that could reverse antimony resistance. This finding also has implications for other diseases, e.g., cancer (28), in which glutathione has been shown to be important in mediating drug resistance.

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