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

K. C. Carter,1* S. Sundar,2 C. Spickett,3 O. C. Pereira,4 and A. B. Mullen5

Department of Immunology,1 Department of Bioscience,3 and Department of Pharmaceutical Sciences,5 University of Strathclyde, Glasgow, United Kingdom; Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varansi, India2; and Faculty of Pharmacy, Porto University, Porto, Portugal4

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Resistence to pentavalent antimonial (Sb³⁺) 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 Sb³⁺/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 mitofenine, paromomycin and amphoterican B, formulation (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 (Sb³⁺) 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 Sb³⁺ susceptible or resistant on the basis of their response to single-dose treatment with SSG at a dose of 300 mg of Sb³⁺/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

* Corresponding author. Mailing address: Department of Immunology, SIBS, University of Strathclyde, 31 Taylor St., Glasgow G4 0NR, United Kingdom. Phone: 0141-552-4400. Fax: 0141-548-3427. E-mail: K.carter@strath.ac.uk.
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 efficiencies 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′(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 tetraethyleneglycol mono-n-hexadecylether was purchased from Chesham Chemicals Ltd., Harrow, United Kingdom. Paromomycin sulphate, 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 (Meriones auratus; Harlan Olac, Bicester, Oxon, United Kingdom) were used for maintenance of the L. donovani strains. L. donovani strains 200011 and 200106, 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 10⁷L. donovani promastigotes (2). The day of parasite administration to the mice was designated day 0 of experiment 2. Treatment with free SSG caused a significant reduction in both splenic (47%; P < 0.01) and liver (86%; 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 20011, 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′ sensitive) compared to those in the controls. SSG treatment also caused a significant (P < 0.05) reduction (67% ± 12%) in splenic parasite burdens of 200011 infected with SSG.
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\(^r\) 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\% \(P < 0.05\) for strain 200016; 53\% \(P < 0.01\) 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\% \(P < 0.04\); liver (97\% \(P < 0.0005\); parasite burdens in mice infected with L. donovani strain 200016 but had no significant effect on bone marrow parasite burdens compared to the controls in the experiments. 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\% \(P < 0.05\); liver, 96\% \(P < 0.005\); bone marrow, 70\% \(P < 0.005\)) (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 \(\pm\) SEM percent suppression for strain 200016, 99.5\% \(P < 0.05\) for the spleen, 100\% \(P < 0.01\) for the liver, and 97.5\% \(P < 0.05\) for the bone marrow; mean \(\pm\) SEM percent suppression for strain 200011, 94.8\% \(P < 0.05\) for the spleen, 99.3\% \(P < 0.005\) for the liver, and 99\% \(P < 0.005\) 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 formula-

<table>
<thead>
<tr>
<th>Expt no., strain, and treatment (dose)</th>
<th>Mean (\pm) SEM % parasite burden (mean (\pm) SEM % suppression)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>Control</td>
<td>36 (\pm) 13</td>
</tr>
<tr>
<td>SSG</td>
<td>40 (\pm) 14 (21 \pm 13)</td>
</tr>
<tr>
<td>Miltefosine, oral (15 mg/kg)</td>
<td>27 (\pm) 6 (27 \pm 15)</td>
</tr>
<tr>
<td>Paromomycin (20 mg/kg)</td>
<td>51 (\pm) 2 (0 \pm 0)</td>
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<tr>
<td>AMB deoxycholate (0.375 mg/kg)</td>
<td>13 (\pm) 2 (65 \pm 4)</td>
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<tr>
<td></td>
<td>2,197 (\pm) 256</td>
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<tr>
<td></td>
<td>329 (\pm) 125</td>
</tr>
<tr>
<td>Control</td>
<td>31 (\pm) 7</td>
</tr>
<tr>
<td>SSG</td>
<td>32 (\pm) 7 (20 \pm 14)</td>
</tr>
<tr>
<td>Miltefosine, oral (15 mg/kg)</td>
<td>17 (\pm) 3 (47 \pm 10)</td>
</tr>
<tr>
<td>Paromomycin (20 mg/kg)</td>
<td>37 (\pm) 7 (8 \pm 8)</td>
</tr>
<tr>
<td>AMB deoxycholate (0.375 mg/kg)</td>
<td>13 (\pm) 6 (59 \pm 19)</td>
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<tr>
<td></td>
<td>99 (\pm) 25 (96 \pm 1)</td>
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<tr>
<td></td>
<td>99 (\pm) 24 (70 \pm 7)</td>
</tr>
<tr>
<td>Control</td>
<td>50 (\pm) 8</td>
</tr>
<tr>
<td>SSG</td>
<td>17 (\pm) 6 (67 \pm 12)</td>
</tr>
<tr>
<td>Liposomal AMB (8 mg/kg)</td>
<td>0.25 (\pm) 0.25 (99.5 \pm 0.5)</td>
</tr>
<tr>
<td>Miltefosine, oral (25 mg/kg)</td>
<td>0.1 (\pm) 0.1 (99.8 \pm 0.2)</td>
</tr>
<tr>
<td>Control</td>
<td>61 (\pm) 11</td>
</tr>
<tr>
<td>SSG</td>
<td>94 (\pm) 15 (2 \pm 2)</td>
</tr>
<tr>
<td>Liposomal AMB (8 mg/kg)</td>
<td>3 (\pm) 2 (94.8 \pm 2.5)</td>
</tr>
<tr>
<td>Miltefosine, oral (25 mg/kg)</td>
<td>1 (\pm) 0.2 (99.8 \pm 0.2)</td>
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<tr>
<td></td>
<td>1,767 (\pm) 191</td>
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<tr>
<td></td>
<td>244 (\pm) 32</td>
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<td></td>
<td>1,913 (\pm) 296 (8 \pm 50)</td>
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<td></td>
<td>298 (\pm) 48 (4 \pm 4)</td>
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<td></td>
<td>10 (\pm) 5 (99.3 \pm 0.4)</td>
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<tr>
<td></td>
<td>2 (\pm) 2 (99 \pm 0.6)</td>
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<tr>
<td></td>
<td>7 (\pm) 4 (99.6 \pm 0.2)</td>
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<tr>
<td></td>
<td>6 (\pm) 3 (97.8 \pm 1.4)</td>
</tr>
</tbody>
</table>

\(^a\) 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\(^r\)/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.
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% sup-
pression 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, treat-
ment 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 (Ta-
ble 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). How-
ever, both total \((P < 0.01)\) and reduced \((P < 0.01)\) levels of

\[\begin{array}{c|c|c|c}
\text{Batch 1} & \text{Batch 2} \\
\hline
\text{200016} & \text{200016} \\
\text{Batch 2} & \text{200011} \\
\text{Control} & \text{Control} \\
\text{SSG (282 mg of \(Sb^v/kg\))} & \text{SSG (70.4 mg of \(Sb^v/kg\))} \\
\text{BSO-NIV} & \text{BSO-NIV} \\
\text{BSO-NIV and SSG} & \text{BSO-NIV and SSG} \\
\end{array}\]

\[\begin{array}{c|c|c|c}
\text{Spleen} & \text{Liver} & \text{Bone marrow} \\
\hline
\text{Control} & 18 ± 5 & 855 ± 102 & 145 ± 28 \\
\text{SSG (282 mg of \(Sb^v/kg\))} & 11 ± 2 (40 ± 14) & 15 ± 6\(a\) (98 ± 1) & 43 ± 15\(b\) (72 ± 9) \\
\text{BSO-NIV} & 38 ± 6\(b\) (0 ± 0) & 850 ± 64 (7 ± 4) & 287 ± 47 (0 ± 0) \\
\text{BSO-NIV and SSG} & 5 ± 1\(c\) (72 ± 7) & 11 ± 8\(c\) (99 ± 1.0) & 11 ± 4\(c\) (97 ± 1) \\
\text{Control} & 10 ± 2 & 567 ± 117 & 101 ± 5 \\
\text{SSG (282 mg of \(Sb^v/kg\))} & 8 ± 2 (30 ± 15) & 476 ± 107 (22 ± 15) & 106 ± 9 (6 ± 6) \\
\text{BSO-NIV} & 14 ± 1 (0 ± 0) & 486 ± 1.267 (13 ± 9) & 75 ± 25 (35 ± 20) \\
\text{BSO-NIV and SSG} & 58 ± 17\(b\) (0 ± 0) & 23 ± 9\(a\) (96 ± 2) & 87 ± 20 (21 ± 16) \\
\text{Control} & 22 ± 4 & 1,681 ± 189 & 305 ± 40 \\
\text{SSG (70.4 mg of \(Sb^v/kg\))} & 30 ± 4 (5 ± 2) & 395 ± 60\(b\) (77 ± 4) & 367 ± 43 (25 ± 20) \\
\text{BSO-NIV} & 34 ± 7 (7 ± 7) & 571 ± 44\(b\) (65 ± 2) & 239 ± 36 (22 ± 12) \\
\text{BSO-NIV and SSG} & 0.5 ± 0.5\(a\) (98 ± 2) & 2 ± 2\(a\) (99.9 ± 0.1) & 82 ± 31\(a\) (73 ± 10) \\
\text{Control} & 58 ± 17 & 2,321 ± 282 & 402 ± 137 \\
\text{SSG (70.4 mg of \(Sb^v/kg\))} & 68 ± 15 (9 ± 11) & 3,066 ± 496 (4 ± 4) & 426 ± 80 (10 ± 7) \\
\text{BSO-NIV} & 53 ± 18 (28 ± 14) & 2,835 ± 207 (0 ± 0) & 459 ± 45 (3 ± 2) \\
\text{BSO-NIV and SSG} & 438 ± 37\(b\) (0 ± 0) & 154 ± 37\(a\) (93 ± 1) & 962 ± 173\(b\) (0 ± 0) \\
\end{array}\]

\[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 \(Sb^v/kg\) at 2× for 5 separate experiments); Table 2). BSO-NIV in mice infected with strain 200011 was not treated with the relevant controls (Table 3). 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\) \(P < 0.01\) compared to the results for the relevant control.

\[c\) \(P < 0.05\) compared to the results for the relevant control.

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, liv-
ers, and bone marrow compared to the numbers in the con-
trols. This effect \((P < 0.001)\) was independent of the batch
of BSO used to prepare the BSO-NIV formulation (in four of
different 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
in the controls (in five of five separate experi-
ments; 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 exper-
iments; Table 2) or caused a significant \((P < 0.001)\) increase in
bone marrow parasite burdens (in four of five separate experi-
ments; 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
showed that the use of SSG at higher doses (>138 mg of SBv/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 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 ± 0.01 g 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 antimonials 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 SBv 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 SBv/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\(^{3+}\)/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 anthimony had accumulated in tissues as the Sb\(^{3+}\) 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\(^{3+}\)/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\(^{3+}\)/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\(^{3+}\) 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 anthimony (Sb\(^{v}\) and Sb\(^{3+}\)) 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 biotin-\(\alpha\)-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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