Antimony Resistance and Trypanothione in Experimentally Selected and Clinical Strains of *Leishmania panamensis*

**ANTIMONY RESISTANCE** in *L. PANAMENSIS*

Diego A. Goyeneche-Patino, Liliana Valderrama, John Walker, Nancy G. Saravia *

Centro Internacional de Entrenamiento e Investigaciones Medicas (CIDEIM), Cali, Colombia.

* Corresponding Author: Centro Internacional de Entrenamiento e Investigaciones Medicas (CIDEIM). Avenida 1 Norte # 3-03, Cali, Colombia.

Telephone: +57 2 6682164. Fax: +57 2 6672989.

e-mail: saravian@cideim.org.co
Abstract

Participation of trypanothione in clinical and experimental Sb resistance in *L. panamensis* was examined using specific inhibitors. BSO significantly reversed resistance to SbIII of promastigotes of experimentally derived Sb resistant lines, supporting the participation of a trypanothione mediated mechanism of resistance. In contrast, promastigotes of strains isolated at clinical treatment failure and resistant to SbV as intracellular amastigotes were not cross resistant to SbIII, and BSO had little or no effect on susceptibility. DFMO did not alter SbIII susceptibility of experimentally selected lines or clinical strains. Mechanisms of acquired resistance emerging in clinical settings may differ from those selected by *in vitro* exposure to antimony.
Antimonials have been the first line treatment for all clinical forms of leishmaniasis for over 60 years (30). Treatment failure increasingly challenges the effective management of leishmaniasis (1, 19, 23, 29). Proof for selection of resistant populations of *Leishmania* during treatment and prospective demonstration of a causal link between treatment failure and drug resistant *Leishmania* have recently been established with clinical isolates of the *Viannia* subgenus (26).

The mechanisms of action of antimonials remain uncertain, however it is generally considered that pentavalent antimony (Sb$^V$) is a prodrug that is reduced to the active trivalent form (Sb$^III$). This reductive activation evidently occurs inside both the parasite and host macrophages (25, 27, 28). While down-regulation of the genes encoding TDR1, AQP1, and ACR2 confers resistance to Sb$^III$ *in vitro* (12, 15, 30), the best known mechanism of experimental resistance to Sb involves detoxification of Sb$^III$ via conjugation to trypanothione (T[SH]$_2$) (13, 14, 18). Overproduction of T[SH]$_2$ results from enhanced capacity of the parasite to produce the precursors of T[SH]$_2$: glutathione (GSH) and spermidine, mediated respectively by amplification of the gsh1 gene, encoding γ-glutamylcysteine-synthetase (γGCS) and by transcriptional over-expression of ornithine decarboxylase (ODC)(16, 17, 21). T[SH]$_2$ synthesis can be interrupted using drugs that inhibit the synthesis of glutathione and spermidine. Inhibition of T[SH]$_2$ synthesis could increase the efficacy of antimonial drugs in the presence or absence of drug resistance.
This study sought to determine whether T[SH]$_2$ is involved in experimental and acquired clinical Sb resistance of *Leishmania panamensis*.

The contribution of T[SH]$_2$ to Sb resistance was determined using lines selected *in vitro* from the parental clone *L. panamensis* 1166 wild type (WT) and paired clinical strains that were Sb$^V$ sensitive when isolated at diagnosis and presented secondary Sb$^V$ resistance at clinical treatment failure of patients treated with the standard regimen of meglumine antimoniate (Table 1).

*L. panamensis* promastigotes were selected for Sb$^{III}$ resistance as described (7). The human promonocytic line U937 was cultured, differentiated to macrophages and infected with promastigotes of the parental antimony sensitive line 1166 (WT) (5). *In vitro* selection of intracellular amastigotes for Sb$^V$ resistance was achieved by culture of infected macrophages in RPMI medium containing an additive-free formulation of Glucantime® (Walter Reed 214975AK, lot N°. BLO9186 90-278-1A1 W601). Amastigote-laden cells were exposed to increasing Sb$^V$ concentration, starting at the 50% effective dose (ED$_{50}$) of the parental clone, (7 µg Sb$^V$/ml), up to 898 µg Sb$^V$/ml. Intracellular amastigotes were passaged twice at each Sb$^V$ concentration.

The effect of inhibitors of T[SH]$_2$ synthesis on parasites, monocytes and macrophages was evaluated independently to determine the appropriate inhibitor concentration to revert Sb resistance. The number of viable cells was measured using acid phosphatase (AP) as described (4). Monocytes and macrophages seeded at $1 \times 10^5$ cells/ml 2 days
prior, were exposed to serial concentrations from 0.063 to 10 mM of BSO (Acros Organics, Morris Plains, New Jersey), DFMO (donated by Dr. Alan Fairlamb) and BSO+DFMO during 48 h. The effect of BSO and DFMO in combination with Sb\textsuperscript{V} on U937 monocytes/macrophages was measured using 1 x 10\textsuperscript{5} cells/ml exposed to 0.25 to 4 mM BSO and/or DFMO followed 24 h later by the addition of 2-128 µg Sb/ml. The number of viable cells was evaluated 72 h later.

Dose-response of log phase (48 hr) Sb\textsuperscript{III}-resistant and sensitive promastigotes (Table 1), at 4 x10\textsuperscript{6}/ml was determined with BSO (0.063 –16 mM), DFMO (0.25 – 6 mM) and BSO+DFMO (each at 0.063 – 8 mM) during 48 h.

The effect of inhibitors of T[SH]\textsubscript{2} synthesis on the ED\textsubscript{50} of Sb\textsuperscript{III} for promastigotes was evaluated in 2 x 10\textsuperscript{6} parasites/ml exposed to 5mM BSO, DFMO and BSO+DFMO during 48 h, then resuspended at 4 x 10\textsuperscript{6} parasites/ml and cultured with 16 to 1024 µgSb\textsuperscript{III} /ml and 0.25 to 32µg Sb\textsuperscript{III} /ml respectively, for resistant and sensitive parasites. Viability index was defined as optical density (OD) of parasites exposed to drugs divided by the OD of untreated parasites.

The ED\textsubscript{50} values of Sb\textsuperscript{III} and Sb\textsuperscript{V} were calculated using the Probit Model (6). Differences between treatments (T[SH]\textsubscript{2} inhibitors plus Sb\textsuperscript{III}, and Sb\textsuperscript{III} alone) were calculated using ANOVA or Kruskal-Wallis according to data distribution. Mean ED\textsubscript{50} values were compared using the Duncan test. \textit{P} values of < 0.05 were considered significant. Statistical analyses were performed on data from at least 3 independent
experiments, each comprising four replicates, using SPSS software (SPSS Inc. Chicago, Illinois).

BSO was non-toxic at \( \geq 5 \text{mM} \) concentrations for WT and Sb\(^{\text{III}}\) resistant promastigotes (viability index \( \geq 95\% \)) whereas 6 mM DFMO reduced the viability index of the Sb\(^{\text{III}}\) resistant lines by 14\% and susceptible strains by 30\%. The toxicity of BSO+DFMO was similar to DFMO alone. Reversion assays were therefore conducted at 5mM BSO and DFMO. FACS analysis of propidium iodide stained promastigotes confirmed the cytostatic activity of BSO and DFMO.

BSO treatment reduced the ED\(_{50}\) of Sb\(^{\text{III}}\) for promastigotes of the resistant \textit{L. panamensis} lines 800.3, 800.5 and 1000.1, by 58.32\%, 62.92\% and 83.61\%. The combination BSO+DFMO similarly reduced the ED\(_{50}\) of Sb\(^{\text{III}}\) by 56.97\%, 50.92\% and 81.13\% respectively (Figure 1A). The reduction in ED\(_{50}\) by BSO or BSO+DFMO was significant for each line: 800.3 (\( P<0.003 \)), 800.5 (\( P<0.001 \)) and 1000.1 (\( P<0.01 \)). DFMO did not significantly alter the ED\(_{50}\) of Sb\(^{\text{III}}\) for these lines.

The resistant line SRA898, selected as intracellular amastigotes using Sb\(^{\text{V}}\), presented a similar pattern of resistance (Table 2) and reversion as lines selected as promastigotes using Sb\(^{\text{III}}\). The ED\(_{50}\) of Sb\(^{\text{III}}\) for promastigotes was reduced by 78\%, 21\% and 86\% when treated with 5 mM BSO, DFMO and BSO+DFMO respectively (Figure 1B); \( P<0.02 \) for BSO and BSO+DFMO versus Sb\(^{\text{III}}\) treatment alone.
Among the 10 clinical strains evaluated, only WT promastigotes demonstrated significant reduction of the ED50 of SbIII (6.2 µg/ml ± SE 0.30) on exposure to BSO (4.9 µg/ml ± SE 0.20) and BSO+DFMO (4.7 µg/ml ± SE 0.15) (P<0.009) Figure 1A, Table 2.

Strains isolated at relapse and resistant to SbV as intracellular amastigotes, were as susceptible to SbIII as promastigotes of the pretreatment SbV sensitive strain (Table 2). There was no correlation between the ED50 values of SbIII for promastigotes and SbV for intracellular amastigotes of clinical strains whereas ED50 values of both SbIII and SbV were consistently high in resistant lines selected in vitro.

Monocytes proved more sensitive to inhibitors of T[SH]2 synthesis than macrophages. BSO reduced the viability index of monocytes by 27.3 % at the highest dose (10mM), but did not affect macrophages. 10mM DFMO reduced the viability index of monocytes by 42% and macrophages by 34.5 %. The combination of BSO+DFMO reduced the viability index of monocytes by 88.2% yet was comparable to exposure to DFMO alone for macrophages.

Reversion of resistance to Sb in intracellular amastigotes was not evaluable because of the high toxicity of SbV in combination with inhibitors of T[SH]2 synthesis. Culture with 1.25mM and 5mM BSO or BSO+DFMO, followed by SbV reduced the viability index of macrophages by 76% and 82% respectively (Figure 1C). DFMO did not significantly increase the toxicity of SbV. ED50 of SbV for macrophages was 858.9 ugSb/mL.
Assessment of drug susceptibility of intracellular pathogens requires consideration of potential interaction of drugs, and toxicity for the host cell. Monocytes were significantly more sensitive to BSO than macrophages. This stage specific effect likely reflects the low replication rate of macrophages compared with monocytes; glutathione is produced in monocytes prior to mitosis and BSO impairs replication (10). Nevertheless, BSO rendered U937 macrophages highly susceptible to Sb$^V$. Consequently, we were unable to evaluate the effect of BSO on Sb susceptibility of intracellular amastigotes. Sb$^V$ alone was toxic for U937 macrophages in this and a prior study (26), imposing an upper limit on evaluable antimony concentration in susceptibility assays.

Partial reversion of Sb$^{III}$ resistance by BSO in experimentally selected parasites (Figure 1A) supports involvement of $\gamma$GCS by increased transcription and translation or gene amplification. ODC evidently did not participate in loss of susceptibility to Sb in these lines. Alternatively, importation of exogenous polyamines (3) or synthesis via secondary pathways like $S$-adenosylmethionine decarboxylase (AdoMetDC) (3, 22) could have obscured overproduction of ODC in Sb resistant lines. Reduction of the ED$_{50}$ of Sb$^{III}$ in WT strain supports the potential of $\gamma$GCS in Sb susceptibility of clinical strains.

Loss of susceptibility to Sb$^{III}$ even after intense in vitro selection with Sb$^{III}$ or Sb$^V$ was not completely attributable to trypanothione mediated detoxification, supporting the participation of other mechanisms. Clinical exposure and acquired resistance to Sb$^V$ did not lead to loss of susceptibility to Sb$^{III}$ in the $L$ panamensis strains evaluated, and inhibition of $T[SH]_2$ synthesis had limited effect on sensitivity to Sb$^{III}$. In another clinical
setting, γGCS, ODC, and AQP1 expression were reduced in Sb resistant *L. donovani* strains in India and Nepal, yet Sb resistance was reverted by BSO (11, 20).

The dichotomy in susceptibility of clinical strains to different oxidation states of antimony contrasts with the cross resistance of experimentally selected resistant lines to Sb\(^V\) as intracellular amastigotes and to Sb\(^{III}\) as promastigotes. Since selection of intracellular amastigotes using Sb\(^V\) *in vitro* led to cross resistance of the promastigote form to Sb\(^{III}\), life stage *per se* does not explain the different outcome of experimental selection with Sb\(^{III}\) or Sb\(^V\) versus treatment with Sb\(^V\). Both cross resistance (20), and absence of correlation between susceptibility to Sb\(^{III}\) and Sb\(^V\) (24) have been reported for clinical strains.

Transitory exposure to low concentrations of Sb during treatment with Glucantime® could select different mechanisms of resistance than *in vitro* selection. During standard treatment of leishmaniasis with Glucantime® peak antimony levels in plasma are 20 – 40 µg/ml (8, 9). *In vitro* selection involves exposure to 10-15 times higher antimony concentrations over several weeks. Levels of antioxidants in *Leishmania* reportedly differ depending on the life stage. *L. donovani* and *L. major* amastigotes have at least 10 fold lower concentrations of thiols, glutathione, trypanothione and ovothiol than promastigotes (2). This may explain the higher intrinsic susceptibility of amastigotes and resistance of promastigotes to Sb\(^V\). Furthermore, host cells can regulate entry and efflux and metabolize drugs targeted to intracellular pathogens. Hence experimental *in vitro* selection may not mimic selection in vivo.
In summary, BSO significantly reverses resistance to antimony in experimentally derived Sb resistant lines of *L. panamensis*. This finding supports the participation of a trypanothione mediated mechanism of resistance in these lines. In contrast, strains isolated at clinical treatment failure and resistant to Sb\textsuperscript{V} as intracellular amastigotes were not cross resistant to the presumptive active form of antimony, Sb\textsuperscript{III}, and BSO had little or no effect on susceptibility. Our results provide evidence that mechanisms of acquired resistance emerging in clinical settings may be different from those selected by *in vitro* exposure to antimony, underscoring the need to clarify the mechanisms involved in clinical resistance.

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Literature Cited


Figure 1.
Figure 1. Effect of inhibitors of T[SH]$_2$ synthesis on the susceptibility of the cloned parental 1166 WT, Sb resistant lines generated by experimental selection in vitro, and U937 macrophages.  (A) ED$_{50}$ of Sb$^{III}$ of promastigotes of lines selected as promastigotes using Sb$^{III}$ (Error bars correspond to mean ± 2 standard errors), (B) Dose response to Sb$^{III}$ of line SRA898, selected as amastigotes using Sb$^V$, (C). Sensitivity of human U937 macrophages to Sb$^V$ alone and with different concentrations of BSO (One representative experiment of 3 independent experiments). Viability Index=Treated cells or parasites/Untreated cells or parasites.

* ANOVA; Duncan, $P< 0.05$: a. Sb$^{III}$ vs. BSO+Sb$^{III}$, b. Sb$^{III}$ vs. BSO+DFMO+Sb$^{III}$

** Kruskal-Wallis, $P=0.026$, differences between Sb$^{III}$ vs. BSO+Sb$^{III}$ and Sb$^{III}$ vs. BSO+DFMO+Sb$^{III}$
Table 1. Strains and lines of *L. panamensis* evaluated in this study.

<table>
<thead>
<tr>
<th>Abbreviated Code</th>
<th>Leishmania panamensis</th>
</tr>
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<tbody>
<tr>
<td>1166 WT</td>
<td>MHOM/COL/86/1166</td>
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</table>

**Selected *in vitro* as promastigotes with Sb\textsuperscript{III}**
- 800.3           | MHOM/COL/86/1166-800.3      |
- 800.5           | MHOM/COL/86/1166-800.5      |
- 1000.1          | MHOM/COL/86/1166-1000.1     |

**Selected *in vitro* as amastigotes with Sb\textsuperscript{V}**
- SRA898          | MHOM/COL/86/1166-SRA898     |

**Clinical Strains (26)**

<table>
<thead>
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<th>Isolated from patients with successful treatment</th>
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<tr>
<td>3332</td>
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<td>4790</td>
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<td>6138</td>
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<table>
<thead>
<tr>
<th>Isolated from patients with clinical treatment failure</th>
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</thead>
<tbody>
<tr>
<td>3594 (I)</td>
</tr>
<tr>
<td>3594 (R1)</td>
</tr>
<tr>
<td>3594 (R2)</td>
</tr>
<tr>
<td>3271 (I)</td>
</tr>
<tr>
<td>3271(R1)</td>
</tr>
<tr>
<td>3278 (I)</td>
</tr>
<tr>
<td>3278(R1)</td>
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</tbody>
</table>

(I) at diagnosis, (R1) first relapse, (R2) second relapse.
Table 2. ED$_{50}$ of Sb$^{\text{III}}$ and Sb$^{\text{V}}$ for clinical strains isolated before treatment and at relapse following treatment with Glucantime® and experimentally selected lines 1000.1 and SRA898. Medians were calculated from 3 or 4 independent experiments.

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Line/Strain</th>
<th>Promastigotes</th>
<th>Intracellular Amastigotes ($\text{ED}_{50}$ µgSb/ml, Median (Minimum, Maximum))</th>
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<tr>
<td></td>
<td></td>
<td>Sb$^{\text{III}}$</td>
<td>Sb$^{\text{III}}$ + BSO 5 mM</td>
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<td><strong>In vitro derived resistant strains</strong></td>
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<tr>
<td>1000.1</td>
<td>Sb$^{\text{III}}$ resistant</td>
<td>109.7 (42.9, 113.8)</td>
<td>17.4. (0.0, 26.3)</td>
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<tr>
<td>SRA898</td>
<td>Sb$^{\text{V}}$ resistant</td>
<td>55.9 (53.9, 74.1)</td>
<td>14.2 (9.6, 15.3)</td>
</tr>
<tr>
<td><strong>Strains isolated from patients</strong></td>
<td></td>
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<tr>
<td>3271</td>
<td>Pre-treatment</td>
<td>4.3 (2.3, 5.8)</td>
<td>4.8 (3.2, 6.9)</td>
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<td>Relapse</td>
<td>4.1 (2.5, 6.2)</td>
<td>4.2 (2.4, 5.1)</td>
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<td>3278</td>
<td>Pre-treatment</td>
<td>3.4 (2.4, 4.5)</td>
<td>3.5 (1.8, 5.5)</td>
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<tr>
<td></td>
<td>Relapse</td>
<td>4.0 (1.6, 7.2)</td>
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<td>3594</td>
<td>Pre-treatment</td>
<td>2.8 (1.8, 5.7)</td>
<td>2.5 (1.5, 3.7)</td>
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<td>Relapse 1</td>
<td>4.2 (4.1, 5.5)</td>
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<td>Relapse 2</td>
<td>5.3 (3.9, 6.0)</td>
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