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

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The participation of trypanothione in clinical and experimental antimony (Sb) resistance in *Leishmania panamensis* was examined by using specific inhibitors. Buthionine sulfoximine (BSO) significantly reversed the resistance to trivalent Sb (Sb^{III}) 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 the time of clinical treatment failure and resistant to pentavalent Sb (Sb^{V}) as intracellular amastigotes were not cross resistant to Sb^{III}, and BSO had little or no effect on susceptibility. Difluoromethylornithine did not alter the Sb^{III} susceptibilities of experimentally selected lines or clinical strains. The mechanisms of acquired resistance emerging in clinical settings may differ from those selected by in vitro exposure to Sb.

Antimonials (Sbs) have been the first line of 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 of the selection of resistant populations of *Leishmania* during treatment and the prospective demonstration of a causal link between treatment failure and drug-resistant *Leishmania* have recently been established with clinical isolates of the subgenus *Viannia* (26).

The mechanisms of action of Sbs 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 the host macrophages (25, 27, 28). While downregulation 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 the detoxification of Sb^{III} via the conjugation to trypanothione [$T(SH)_2$] (13, 14, 18). The overproduction of $T(SH)_2$ results from an enhanced capacity of the parasite to produce the precursors of $T(SH)_2$, glutathione and spermidine, mediated by amplification of the *gsh1* gene, which encodes γ-glutamylcysteine synthetase (γ-GCS), and by the transcriptional overexpression of ornithine decarboxylase (ODC), respectively (16, 17, 21). $T(SH)_2$ synthesis can be interrupted with drugs that inhibit the synthesis of glutathione and spermidine. Inhibition of $T(SH)_2$ synthesis could increase the efficacies of antimonial drugs in the presence or absence of drug resistance.

This study sought to determine whether $T(SH)_2$ is involved in the experimental and acquired clinical Sb resistance of *Leishmania panamensis*.

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

L. panamensis promastigotes were selected for Sb^{III} resistance as described previously (7). The human promonocytic line U937 was cultured, differentiated to macrophages, and infected with promastigotes of the parental Sb-sensitive line 1166 WT (5). The 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 meglumine antimonate (Glucantime; lot BLO9186 90-278-1A1 W601; Walter Reed 214975AK). Amastigote-laden cells were exposed to increasing Sb^{V} concentrations, starting at the 50% effective dose (ED_{50}) for the parental clone (7 μg Sb^{V}/ml) and increasing to 898 μg Sb^{V}/ml. Intracellular amastigotes were passaged twice in the presence of each Sb^{V} concentration.

The effects of inhibitors of $T(SH)_2$ synthesis on parasites, monocytes, and macrophages were evaluated independently to determine the appropriate inhibitor concentration for reversion to Sb resistance. The number of viable cells was measured by using acid phosphatase, as described previously (4). Monocytes and macrophages that had been seeded at 1×10^5 cells/ml 2 days earlier were exposed to serial concentrations ranging from 0.063 to 10 mM of buthionine sulfoximine (BSO; Acros Organics, Morris Plains, NJ), difluoromethylornithine (DFMO; donated by Alan Fairlamb), and BSO-DFMO for 48 h. The effect of BSO and DFMO in combination with Sb^{V} on U937 monocytes/macrophages was measured by using 1×10^5 cells/ml exposed to 0.25 to 4 mM BSO and/or DFMO, followed 24 h later by the addition of 2 to 128 μg Sb/ml. The number of viable cells was evaluated 72 h later.

The dose-responses of log-phase (cultured for 48 h) Sb^{III}-resistant and -sensitive promastigotes (Table 1) at 4×10^6/ml were determined with BSO (0.063 to 16 mM), DFMO (0.25 to 6 mM), and BSO-DFMO (each at 0.063 to 8 mM) for 48 h.

The effects of inhibitors of $T(SH)_2$ synthesis on the ED_{50} of

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TABLE 1. Strains and lines of *L. panamensis* evaluated in this study

Strain group, abbreviated code	*Leishmania panamensis* strain
1166 WT	MHOM/COL/86/1166
Selected in vitro as promastigotes with Sb^{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^{V}, SRA898	MHOM/COL/86/1166-SRA898
Clinical strains (n = 26)	
Isolated from patients with successful treatment	
3332	MHOM/COL/99/3332
4790	MHOM/COL/96/4790
6138	MHOM/COL/02/6138
Isolated from patients with clinical treatment failure[a]	
3594 (I)	MHOM/COL/02/3594
3594 (R1)	MHOM/COL/02/3594-R1
3594 (R2)	MHOM/COL/02/3594-R2
3271 (I)	MHOM/COL/98/3271
3271 (R1)	MHOM/COL/02/3594-R1
3278 (I)	MHOM/COL/98/3278
3278 (R1)	MHOM/COL/98/3278-R1

[a] I, isolated at time of diagnosis; R1, isolated at time of first relapse; R2, isolated at time of second relapse.

Sb^{III} for promastigotes were evaluated with 2×10^6 parasites/ml exposed to 5 mM BSO, DFMO, and BSO-DFMO for 48 h and then resuspended at 4×10^6 parasites/ml and cultured with 16 to 1,024 μg Sb^{III}/ml and 0.25 to 32 μg Sb^{III}/ml for resistant and sensitive parasites, respectively. The viability index was defined as the optical density (OD) of parasites exposed to drugs divided by the OD of untreated parasites.

The ED_{50}s of Sb^{III} and Sb^{V} were calculated by using the probit model (6). Differences between treatments [$T(SH)_2$inhibitors plus Sb^{III} and Sb^{III} alone] were calculated by analysis of variance (ANOVA) or the Kruskal-Wallis test according to the data distribution. Mean ED_{50}s were compared by use of the Duncan test. P values of <0.05 were considered significant. Statistical analyses were performed with data from at least three independent experiments, each comprising four replicates, with SPSS software (SPSS Inc., Chicago, IL).

BSO was nontoxic at concentrations of ≥5 mM for WT and Sb^{III}-resistant promastigotes (viability index ≥ 95%), whereas 6 mM DFMO reduced the viability index of the Sb^{III}-resistant lines by 14% and that of the susceptible strains by 30%. The toxicity of BSO-DFMO was similar to that of DFMO alone. Reversion assays were therefore conducted with 5 mM BSO and DFMO. Fluorescence-activated cell sorter analysis of propidium iodide-stained promastigotes confirmed the cytostatic activity of BSO and DFMO.

BSO treatment reduced the ED_{50}s of Sb^{III} for promastigotes of the resistant *L. panamensis* lines 800.3, 800.5, and 1000.1 by 58.32%, 62.92%, and 83.61%, respectively. The combination BSO-DFMO similarly reduced the ED_{50}s of Sb^{III} by 56.97%, 50.92%, and 81.13%, respectively (Fig. 1A). The reductions in the ED_{50}s by BSO or BSO-DFMO were significant for each cell line: $P < 0.003$ for 800.3, $P < 0.001$ for 800.5, and $P < 0.01$ for 1000.1. DFMO did not significantly alter the ED_{50}s of Sb^{III} for these lines.

Resistant line SRA898, selected as intracellular amastigotes by using Sb^{V}, presented a similar pattern of resistance (Table 2) and reversion as the lines selected as promastigotes with Sb^{III}. The ED_{50}s of Sb^{III} for promastigotes were reduced by 78%, 21%, and 86% when they were treated with 5 mM BSO, DFMO, and BSO-DFMO, respectively (Fig. 1B) ($P < 0.02$ for

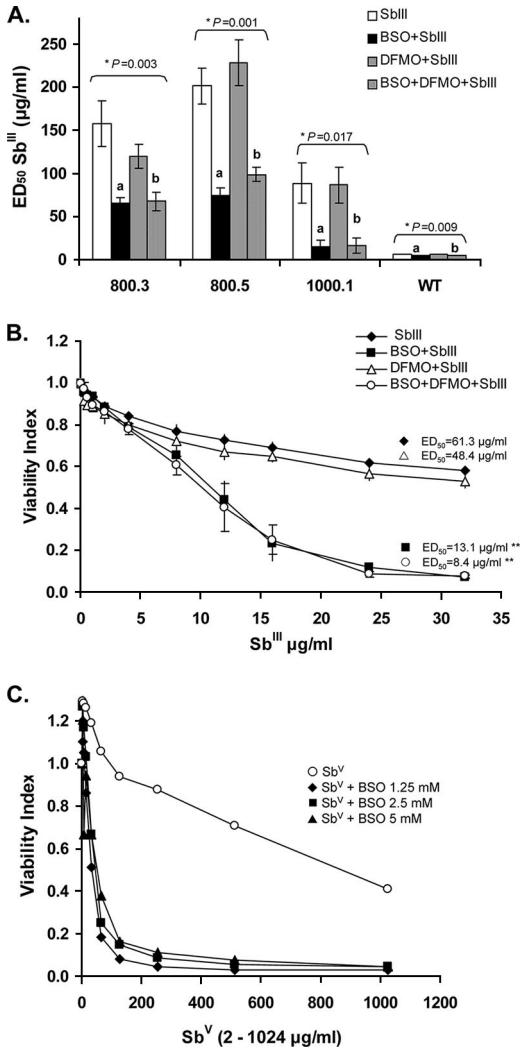

FIG. 1. Effects of inhibitors of $T(SH)_2$ synthesis on the susceptibility of the cloned parental 1166 WT, Sb-resistant lines generated by experimental selection in vitro, and U937 macrophages. (A) ED_{50}s of Sb^{III} for promastigotes of lines selected as promastigotes by using Sb^{III} (error bars correspond to the mean ± 2 standard errors); (B) dose-response to Sb^{III} of line SRA898, selected as amastigotes with Sb^{V}; (C) sensitivity of human U937 macrophages to Sb^{V}. alone and with different concentrations of BSO (the results of one representative experiment of three independent experiments performed are shown). The results of the statistical analyses are as follows: *, ANOVA, Duncan, $P < 0.05$ for Sb^{III} versus BSO-Sb^{III} (a) and Sb^{III} versus BSO-DFMO-Sb^{III} (b); **, Kruskal-Wallis, $P = 0.026$ for differences between Sb^{III} versus BSO-Sb^{III} and Sb^{III} versus BSO-DFMO-Sb^{III}.

TABLE 2. ED_{50}s of Sb^{III} and Sb^{V} for clinical strains isolated before treatment and at relapse following treatment with meglumine antimonate and experimentally selected lines 1000.1 and SRA898

Strain type and cell line	Strain characteristic	Median[a] (minimum, maximum) ED_{50} (μg Sb/ml)		
		Promastigotes		Intracellular amastigotes, Sb^{V} (26)
		Sb^{III}	Sb^{III} + BSO (5 mM)	
In vitro-derived resistant strains				
1000.1	Sb^{III} resistant	109.7 (42.9, 113.8)	17.4 (0.0, 26.3)	>128.0
SRA898	Sb^{V} resistant	55.9 (53.9, 74.1)	14.2 (9.6, 15.3)	>128.0
Strains isolated from patients				
3271	Pretreatment	4.3 (2.3, 5.8)	4.8 (3.2, 6.9)	14.9
	Relapse	4.1 (2.5, 6.2)	4.2 (2.4, 5.1)	>128.0
3278	Pretreatment	3.4 (2.4, 4.5)	3.5 (1.8, 5.5)	14.4
	Relapse	4.0 (1.6, 7.2)	3.1 (1.1, 4.4)	>128.0
3594	Pretreatment	2.8 (1.8, 5.7)	2.5 (1.5, 3.7)	7.6
	Relapse 1	4.2 (4.1, 5.5)	3.8 (2.9, 3.9)	>128.0
	Relapse 2	5.3 (3.9, 6.0)	3.7 (3.3, 4.2)	>128.0

[a] Medians were calculated from three or four independent experiments.

BSO and BSO-DFMO treatment versus Sb^{III} treatment alone).

Among the 10 clinical strains evaluated, only WT promastigotes demonstrated a significant reduction in the ED_{50} of Sb^{III} (6.2 μg/ml ± 0.30 [standard error]) on exposure to BSO (4.9 μg/ml ± 0.20) and BSO-DFMO (4.7 μg/ml ± 0.15) ($P < 0.009$) (Fig. 1A; Table 2).

Strains that were isolated at the time of relapse and that were resistant to Sb^{V} as intracellular amastigotes were as susceptible to Sb^{III} as promastigotes of the pretreatment Sb^{V}-sensitive strain (Table 2). There was no correlation between the ED_{50}s of Sb^{III} for promastigotes and the ED_{50}s of Sb^{V} for intracellular amastigotes of clinical strains, whereas the ED_{50}s of both Sb^{III} and Sb^{V} were consistently high for 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 tested (10 mM) but did not affect macrophages. DFMO at 10 mM reduced the viability index of monocytes by 42% and that of macrophages by 34.5%. The combination of BSO-DFMO reduced the viability index of monocytes by 88.2%; for macrophages, the viability index after exposure to BSO-DMFO was comparable to DMFO alone.

The reversion of resistance to Sb in intracellular amastigotes was not evaluable because of the high toxicity of Sb^{V} for the host cell when it was used in combination with inhibitors of $T(SH)_2$ synthesis. Culture with 1.25 mM and 5 mM BSO or BSO-DFMO, followed by the addition of Sb^{V}, reduced the viability indices of macrophages by 76% and 82%, respectively (Fig. 1C). DFMO did not significantly increase the toxicity of Sb^{V}. The ED_{50} of Sb^{V} for macrophages was 858.9 μg Sb/ml.

Assessment of the drug susceptibilities of intracellular pathogens requires consideration of the 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 lower replication rate of macrophages compared with that of 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 the Sb susceptibilities of intracellular amastigotes. Sb^{V} alone was toxic for U937 macrophages in this study and a prior study (26), imposing an upper limit on the Sb concentration evaluable in susceptibility assays.

The partial reversion of Sb^{III} resistance by BSO in experimentally selected parasites (Fig. 1A) supports the involvement of γ-GCS by increased transcription and translation or gene amplification. ODC evidently did not participate in the loss of susceptibility to Sb in these lines. Alternatively, the importation of exogenous polyamines (3) or synthesis via secondary pathways like the *S*-adenosylmethionine decarboxylase pathway (3, 22) could have obscured the overproduction of ODC in Sb-resistant lines. The reduction of the ED_{50} of Sb^{III} for the WT strain supports the potential role of γ-GCS in the Sb susceptibilities of clinical strains.

The loss of susceptibility to Sb^{III} even after intense in vitro selection with Sb^{III} or Sb^{V} was not completely attributable to $T(SH)_2$-mediated detoxification, supporting the participation of other mechanisms. Clinical exposure and the acquisition of resistance to Sb^{V} did not lead to a loss of susceptibility to Sb^{III} in the *L. panamensis* strains evaluated, and the inhibition of $T(SH)_2$ synthesis had a limited effect on sensitivity to Sb^{III}. In another clinical setting, γ-GCS, 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 the susceptibilities of clinical strains to different oxidation states of Sb contrasts with the cross-resistance of experimentally selected resistant lines to Sb^{V} as intracellular amastigotes and to Sb^{III} as promastigotes. Since the selection of intracellular amastigotes with Sb^{V} in vitro led to the cross-resistance of the promastigote form to Sb^{III}, the life stage per se does not explain the different outcomes of experimental selection with Sb^{III} or Sb^{V} versus treatment with Sb^{V}. Both cross-resistance (20) and the absence of a 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 meglumine antimonate could select for mechanisms of resistance different from those achieved by in vitro selection. During standard treatment of leishmaniasis with meglumine antimonate, peak Sb levels in plasma are 20 to 40 μg/ml (8, 9). In vitro selection involves exposure to Sb concentrations 10 to 15 times higher over several weeks. The 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, $T(SH)_2$, and ovothiol than promastigotes (2). This may explain the higher intrinsic susceptibility of amastigotes and the 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 the resistance to Sb in experimentally derived Sb-resistant lines of *L. panamensis*. This finding supports the participation of a $T(SH)_2$-mediated mechanism of resistance in these lines. In contrast, strains isolated at the time of clinical treatment failure and resistant to Sb^{V} as intracellular amastigotes were not cross resistant to the presumptive active form of Sb (Sb^{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 Sb, underscoring the need to clarify the mechanisms involved in clinical resistance.

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