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Elevated levels of tryparedoxin peroxidase in antimony unresponsive *Leishmania donovani* field isolates

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Abstract

Enhancement of the anti-oxidant metabolism of *Leishmania* parasites, dependent upon the unique dithiol trypanothione, has been implicated in laboratory-generated antimony resistance. Here, the role of the trypanothione-dependent anti-oxidant pathway is studied in antimony-resistant clinical isolates. Elevated levels of tryparedoxin and tryparedoxin peroxidase, key enzymes in hydroperoxide detoxification, were observed in antimonial resistant parasites resulting in an increased metabolism of peroxides. These data suggest that enhanced anti-oxidant defences may play significant in clinical resistance to antimonials.

Keywords

antimonial resistance; *Leishmania donovani*; tryparedoxin peroxidase

After more than half a century as the mainstay therapy for leishmaniasis, the clinical value of antimonial drugs is threatened by the emergence of acquired drug resistance [1]. In recent years, failure of antimony-based therapies in Bihar, India, has escalated, with over 50% of the estimated 250,000 annual cases of visceral leishmaniasis (VL) being unresponsive. A comprehensive understanding of the molecular mechanisms of resistance to antimonials is crucial, not only in the on-going fight to save these therapies, but also for future design of more effective anti-leishmanials.

To date, our knowledge of antimony resistance mechanisms in *Leishmania spp.* has primarily emerged from the study of laboratory-generated drug-resistant cell lines, created through step-wise exposure to antimony [2, 3]. In more recent times, several features of drug resistance have been corroborated in antimonial-resistant field isolates and have placed the unique thiol metabolism of *Leishmania* at the centre of antimonial resistance mechanisms [4-6]. In *Leishmania*, the major low molecular mass thiol is trypanothione (T[SH]₂ or N¹,N⁸-bis(glutathionyl)spermidine), in contrast to most other organisms which utilize glutathione (GSH or γ-L-glutamyl-L-cysteinylglycine) [7]. In laboratory-induced

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antimonial-resistant strains of *Leishmania*, elevated levels of T[SH]₂ and GSH, following over-expression of the rate-limiting enzymes involved in the synthesis of GSH (γ -glutamylcysteine synthase, 8) and polyamines (ornithine decarboxylase, 9) have been observed. In addition, overexpression of PgpA, an intracellular metal-thiol transporter from the ATP-binding cassette transporter family, has also been demonstrated to play a key role in resistance [8, 10]. Since T[SH]₂ is known to conjugate with the reduced form of antimony (Sb^{III}) [11], it has been hypothesised that antimony is detoxified via the formation of Sb^{III}-thiol complexes, which then get sequestered into an intracellular vacuolar compartment by PgpA [10].

One of the principal functions of T[SH]₂ within *Leishmania* is to provide reducing equivalents to facilitate the parasite's defence against oxidative stress [7, 12] with enzymes of the trypanothione peroxidase family considered to be principally responsible for detoxification of peroxides [13]. The decameric type I trypanothione peroxidase (TryP) [14, 15], is a 2-Cys peroxidase, obtaining its reducing equivalents from T[SH]₂ via the dithiol protein trypanothione (TryX). Studies have associated overexpression of TryP with resistance to both arsenite [16], and antimony [17] in laboratory-generated *Leishmania* resistant cell lines implying that enhanced antioxidant defences, through overexpression of TryP, may well be a key feature of resistance to antimonials in field isolates.

To test this hypothesis, clinical isolates were derived from patients with VL who either responded to sodium stibogluconate (Sb^V, SAG) (S1 and S2) or were refractory to SAG (R1, R2 and R3) and cultured as previously described [6]. In addition, isolates were obtained from patients with VL who came from a SAG responsive zone (S3) or areas having mixed or high resistance to SAG (R4 and R5) [18]. To verify their resistance phenotype, a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay was used to determine their relative sensitivities to Sb^{III} in promastigotes [19]. In addition, isolates were also assayed for their sensitivity to Sb^V in amastigotes using a previously described murine macrophage assay [4]. In line with their clinical designation, isolates resistant to antimonials or from an antimonial-unresponsive zone (R1-5, Sb^R) were markedly less sensitive to both Sb^{III} and Sb^V in both developmental stages than their clinically sensitive counterparts (S1-3, Sb^S, Table 1).

To establish a correlation, if any, between elevated TryP and clinical resistance to antimonials, immunoblots of whole cell lysates were probed with antisera against TryP and TryX, as previously described [17]. In keeping with our previous studies of lab-generated Sb^{III} resistance, TryP was found to be clearly amplified at the protein level in all Sb^R strains as compared to their Sb^S counterparts (Figure 1). In addition, levels of TryX, important in controlling the flux of reducing equivalents from T[SH]₂ to TryP, were also markedly elevated in three out of five resistant isolates (R1, R2 and R3, Figure 1). To examine whether the overexpression of TryP and TryX in Sb^R isolates correlated with an enhanced ability to metabolise hydroperoxides, clarified extracts were prepared and assayed for T[SH]₂-dependent peroxidase activity, H₂O₂ being the substrate (Table 2, 17). All Sb^R isolates studied were found to metabolise H₂O₂ at higher rates than Sb^S isolates, with mean specific activities of 10.6 ± 2.8 and 3.8 ± 1.8 nmol min⁻¹ mg⁻¹, respectively. In particular, lysate prepared from isolate R2 was found to metabolise H₂O₂ most efficiently with its specific activity (13.1 ± 0.8 nmol min⁻¹ mg⁻¹) being 2.4, 3.0 and 7-fold higher than activities of S1, S2 and S3 lysates, respectively. In contrast, lysate derived from isolate R3 was the least adept of all Sb^R isolates at metabolising H₂O₂, being only marginally more efficient than S1. On an individual basis, the activities of R1, R2, R4 and R5 were significantly higher than S1 ($p < 0.001$), S2 ($p < 0.001$) and S3 ($p < 0.001$), whereas the activity of R3 was significantly higher than S2 ($p < 0.05$) and S3 ($p < 0.001$). To ensure that cells had been adequately extracted, the specific activity of alanine aminotransferase (ALAT), an

unrelated housekeeping enzyme was monitored. Reassuringly, ALAT activity was similar in all lysates derived from both Sb^R and Sb^S field isolates. As in the case of laboratory-generated Sb^{III}-resistant cells [17], metabolism of H₂O₂ in Sb^R isolates was further enhanced upon addition of exogenous recombinant *L. major* TryX (Table 2). These observations suggest that TryX remains the rate-limiting step of peroxide metabolism, under these assay conditions.

Trypanothione reductase (TryR) is considered as a key enzyme in the redox metabolism of *Leishmania* since it acts as the sole route of reducing equivalents from the NADP⁺/NADPH couple to T[SH]₂ [20]. Indeed, amplification of TryR has been associated with antimony resistance in field isolates [5]. To examine its overexpression in the antimony resistance mechanisms of our clinical isolates, TryR activity was monitored (Table 2, 20). TryR activity was relatively similar across all Sb^S and Sb^R extracts assayed, except for R1 which maintained an elevated reductase activity (~2-3-fold). The absence of a direct correlation between elevated TryR activity and antimonial resistance indicates that at least in the clinical setting, no clear role can be attributed to TryR and analysis of additional isolates is necessary.

As illustrated in several recent studies, clinical resistance to antimonial drugs is undoubtedly a multi-factorial process and contributory factors include elevated intracellular thiols [5,6], overexpression of drug efflux pumps (Pgp A) [4] and modulation of drug uptake transporters (aquaglyceroporin) [21]. In the current study, we add to this growing list by demonstrating a strong association between antimony resistance in a clinical setting and an enhanced ability to metabolize hydrogen peroxide. Since exposure to antimonials is known to lead to accumulation of damaging reactive oxygen species within *Leishmania* parasites [6, 11], it is easy to envisage how an enhanced ability to detoxify these deadly metabolites confers resistance in clinical isolates. The exact basis for the elevated levels of TryX and TryP in these clinical isolates remains to be determined, however, it is likely to be due to gene amplification.

As demonstrated in this and other studies [4-6, 11], the individual and cumulative impact of each resistance factor seems to be highly variable in clinical isolates and thus identifying a single biomarker for antimonial resistance is likely to be problematic. Undoubtedly, subsequent studies will be required to further unravel the complexities of clinical resistance. However, here we confirm our earlier *in vitro* observations by demonstrating a clear association between enhanced anti-oxidant defenses and clinical resistance to antimonial drugs.

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Figure 1. Immunoblot analysis of whole cell lysates of antimony sensitive and resistant clinical isolates

Blots of cell extracts from antimony sensitive (S1-3) and antimony-resistant (R1-5) isolates (1×10^7 parasites in each lane) were probed with anti-serum to *L. major* tryparedoxin peroxidase type I (TryP), *L. major* tryparedoxin (TryX) and *L. major* PTR1, as previously described (17).

Table 1
Antimony sensitivity profiles of *L. donovani* clinical isolates

Isolate	Location (Bihar) Degree of resistance	EC ₅₀ Promastigotes (Sb ^{III} , µg ml ⁻¹)	EC ₅₀ Amastigotes (Sb ^V , µg ml ⁻¹)
S1	Responded to SAG	15.0 ± 2.5 ^[19]	9.0 ± 0.5 ^[4]
S2	Responded to SAG	21.3 ± 2.8 ^[19]	13.0 ± 1.5 ^[4]
S3	Siwan (no resistance)	5.8 ± 2.4	7.8 ± 2.5
R1	Non-responsive to SAG	76.1 ± 4.8 ^[19]	24 ± 1.4 ^[4]
R2	Non-responsive to SAG	75.3 ± 5.4 ^[19]	65 ± 3.4 ^[4]
R3	Non-responsive to SAG	111.2 ± 5.4 ^[19]	>100 ^[4]
R4	Mixed resistance (Chapra, Saran)	92.7 ± 5.4	ND
R5?	High resistance (Samastipur)	88.8 ± 7.9	29.3 ± 6.4

The sensitivity of clinical isolates to trivalent antimony (Sb^{III}) in promastigotes was measured by exposing log-phase parasites to varying concentrations of Sb^{III} (0-200 µg ml⁻¹) for 48 h and cell viability assessed by the MTT assay [19]. Amastigote sensitivity to Sb^V [4] was determined in a murine macrophage model. EC₅₀ values are expressed as the mean ± standard deviation of at least two independent experiments.

Table 2
Specific activities of trypanothione reductase and T[SH]₂-dependent peroxidase in soluble extracts of *L. donovani* clinical isolates

Enzymatic activity	Substrate	Specific activity (nmol min ⁻¹ mg ⁻¹)								
		S1	S2	S3	R1	R2	R3	R4	R5	
Trypanothione-dependent peroxidase	H ₂ O ₂	5.5 ± 0.5	4.2 ± 0.4	1.82 ± 0.1	10.8 ± 1.2	13.1 ± 0.8	6.0 ± 0.1	12.7 ± 0.7	10.6 ± 0.3	
	H ₂ O ₂ + TryX (1 μM)	6.8 ± 0.6	6.0 ± 0.2	2.9 ± 0.2	17.2 ± 0.9	18.9 ± 1.2	10.2 ± 1.0	17.7 ± 1.4	13.9 ± 0.8	
Trypanothione reductase	T[SH] ₂	48.3 ± 6.1	35.3 ± 0.8	46.1 ± 1.0	103 ± 3.7	37.5 ± 2.1	38.2 ± 2.1	42.1 ± 1.5	51.7 ± 2.4	
Alanine aminotransferase	Ala + α KG	1570 ± 60	1630 ± 60	1630 ± 100	1640 ± 50	1480 ± 60	1600 ± 20	1680 ± 10	1672 ± 60	

All enzymatic activities were assayed as previously described [17] and corrected for non-enzymatic background rates.