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Role of ABC transporter MRPA, γ-glutamylcysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of *Leishmania donovani*

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***Objectives*: The resistance of clinical isolates of *Leishmania donovani* to sodium antimony gluconate (SAG), the mainstay of treatment in Indian visceral leishmaniasis, has become a critical issue in India. The present work investigates the mechanism of resistance to SAG in parasites isolated from patients who are unresponsive to SAG.**

***Methods and results*: Susceptibility to SAG as determined *in vitro* with intracellular amastigotes correlated well with the clinical response. The ABC transporter gene *MRPA* was amplified in resistant field isolates as part of an extrachromosomal circle. Co-amplification of the pterin reductase gene (*PTR1*) and *MRPA* suggests amplification of the H locus in SAG-resistant isolates. Amplification of *MRPA* was correlated to increased RNA as determined by real-time PCR. MRPA is an ABC-thiol transporter, and cysteine and glutathione were increased in the resistant isolates. Ornithine decarboxylase (a rate limiting enzyme in polyamine biosynthesis), and γ-glutamylcysteine synthetase (a rate limiting enzyme in glutathione biosynthesis), the two building blocks of the main cellular thiol trypanothione, were overexpressed in some of the resistant isolates.**

***Conclusions*: A variety of resistance mechanisms to SAG, most of them consistent with a model based on the study of resistance *in vitro*, were present in clinical isolates from the same geographical region.**

Keywords: drug resistance, sodium antimony gluconate, *Leishmania donovani*

Introduction

Leishmaniasis is caused by a protozoan parasite that gives rise to a wide spectrum of diseases, ranging from the simple self-limiting cutaneous form to the debilitating visceral form, which is often fatal if left untreated. Although pentavalent antimonials like sodium antimony gluconate (SAG) are the age-old conventional therapy for visceral leishmaniasis (VL), in more recent times, increasing resistance to SAG has emerged as a major barrier in the treatment of VL.[1] There has been an epidemic of primary resistance to antimonial drugs in parts of India[1] and an urgent need exists to define the mechanism(s) of resistance.

The precise mechanism of action of SAG remains an enigma but it is generally agreed that pentavalent Sb(V) is reduced to trivalent antimony Sb(III), which constitutes the active form of the drug against the parasite.[2] Reduction of the metal may take place either in the parasites[3–6] or in the macrophages,[7] or in both. To date, work on antimony resistance in *Leishmania* spp. has been mostly on laboratory mutants, in which resistance was induced *in vitro* in the presence of antimony or to the related metal arsenic.[8–10] A consistent resistance mechanism deduced from *in vitro* work involves multiple steps where overproduction of ornithine decarboxylase (ODC), the rate limiting enzyme of the polyamine biosynthetic pathway[11] and γ-glutamylcysteine synthetase (γGCS), the rate limiting enzyme of glutathione (GSH) biosynthesis[8,12] leads to overproduction of trypanothione [$T(SH)_2$],[8,10] which is the major reduced thiol of *Leishmania* and composed of a N^1,N^8-bisglutathione spermidine conjugate.[13] Trypanothione is thought to bind to the active reduced form of the metal[14] and these metal–trypanothione conjugates are either

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sequestered into an intracellular organelle by the ABC transporter, MRPA[15] (formerly known as P-glycoprotein A, PGPA) or extruded outside the cell by an efflux pump.[16] Resistance in field isolates may also involve amplification of genes other than the above-described metabolic pathway.[17] Yet another mechanism leading to down-regulation of an uptake system is the loss of an aquaglyceroporin (AQP1) allele that has been reported to cause an increase in resistance to SAG.[18,19]

Resistance to SAG in field isolates is less well defined, and only recently were susceptibility values, determined using *in vitro* assays, found to correlate with the clinical response.[20–22] We found that several of the findings elucidated while studying resistance in laboratory strains are also operational in field isolates. The mechanism(s) of resistance to SAG varied in isolates from the same geographical area. Understanding the mechanisms of antimony resistance in field isolates of *Leishmania donovani* will aid in development of strategies to avoid or overcome drug resistance.

Materials and methods

Parasite and culture conditions

Promastigotes of Indian *Leishmania donovani* clones, GE1 (MHOM/IN/80/GE1F8R),[23] AG83 (MHOM/IN/80/AG83) and four untyped clonal strains 2001, 41, NS2 and CK2 were isolated from patients with VL and were routinely cultured at 22°C in M-199 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Life Technologies Scotland, UK) and 0.13 mg/mL penicillin and streptomycin.

Clinical isolates obtained prior to drug treatment from VL patients who had responded to SAG chemotherapy were designated as SAG-S (SAG-sensitive), whereas VL patients who did not respond to SAG were designated as SAG-R (SAG-resistant). Accordingly, SAG-S isolates used in this study include AG83-S and 2001-S, whereas the four SAG-R isolates were 41-R, GE1-R, NS2-R, and CK2-R. The SAG-R isolates were maintained in the absence of drug pressure *in vitro*. The isolates have been passaged through hamsters or BALB/c mice to retain their virulence, and importantly, their chemosensitivity profiles have remained unchanged as measured periodically by amastigote-macrophage infectivity assay described below.

DNA construct and transfection

An episomal *Leishmania* expression vector (pGL-αNEOαLUC) containing luciferase-encoding DNA and a neomycin phosphotransferase selectable marker[24] was used in the present study.

The construct (20 μg) was transfected into *L. donovani* promastigotes by electroporation in 2 mm gap cuvettes at 450 V, 500 μF (BTX Electro Cell Manipulator 600). Transfectants were selected for resistance to G418 (50 μg/mL) as described previously.[25,26]

Chemosensitivity profiles of SAG-S and SAG-R strains in a macrophage model

Stationary phase *Leishmania* promastigotes expressing the luciferase gene (pGL-αNEOαLUC) were used to infect J774A.1 macrophages. Macrophage cell line J774A.1 (American Type Culture Collection) was maintained at 37°C in RPMI-1640 medium (Sigma) containing 10% heat inactivated FBS as described previously.[27] Briefly, J774A.1 murine macrophages (1×10^5 cells/250 μL/well) were infected with 1×10^6 promastigotes in M199 media with 10% FBS. After 3 h, the non-internalized parasites were washed off and SAG was added at different concentrations (10–100 μg/mL). After 5 days of drug exposure, plates containing adherent macrophages were washed and luciferase activity was determined.[24] The 50% inhibitory concentration (IC_{50}) was determined from the graph representing different concentrations of SAG plotted against relative light units (RLU) produced by luciferase-expressing parasites.

Nucleic acid isolation, PFGE and hybridization analysis

Genomic DNA was isolated from $\sim 2 \times 10^9$ promastigotes by a standard procedure,[28] and circular DNA was isolated by Promega Wizard miniprep kit following the manufacturer's instructions. Genomic DNA and circular DNA were digested with *Hin*dIII and subjected to electrophoresis in 1% agarose gels. The fragments were transferred to Hybond^{TM} − N + membrane (Amersham Pharmacia Biotech) and subjected to Southern-blot analysis.

Total RNA was isolated from 2×10^8 promastigotes using TRI Reagent^{TM} (Sigma). For northern-blot analysis, 15 μg of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred onto nylon membrane following standard procedures.

Chromosomes of the clinical isolates were separated by PFGE in which low melting agarose blocks, containing embedded cells (10^8 log phase promastigotes per mL) were electrophoresed in a contour clamped homogeneous electric field apparatus (CHEF DRIII, Bio-Rad) in 0.5× Tris-borate-EDTA, with buffer circulation at a constant temperature of 14°C and run time of 24 h, using *Saccharomyces cerevisiae* chromosomes as size markers.

Following the transfer of DNA, RNA and chromosomes on to nylon membranes, the nucleic acids were cross-linked to the membrane with ultraviolet light in a Stratagene UV cross-linker. The blots were hybridized with denatured [α-^{32}P]dCTP-labelled DNA probe. The DNA probes used in the present study included a 400 bp *MRPA* fragment (released from plasmid PM12 that was digested with *Bam*HI and *Pst*I), a 2.3 kb fragment *GSH1* probe (derived from plasmid pspαhygroα-γGCS digested with *Hin*dIII and *Xba*I), a 2 kb *ODC* full-length probe (derived from plasmid pspαhygro-*L. donovani* ODC by digesting with *Hin*dIII and *Xba*I) and a 1.6 kb 5′-*PTR1* probe (derived from plasmid psp72-Y-hygro-5′-PTR1).

Real-time PCR

The human leukaemia cell line THP-1 was infected with promastigotes of *L. donovani* at a ratio of 15 : 1 as described earlier[21,29] and RNAs from intracellular parasites were obtained as described by Decuypere *et al.*[30] Quality and quantity of the RNA were determined using the RNA 6000 NanoLabchip kit on the Bioanalyzer 2100 (Agilent Technologies). The sequences of the primers for MRPA are forward 5′-GCGCAGCCGTTTGT GCTTGTGG and reverse 5′-TTGCCGTACGTCGCGATGGT GC, and for the GAPDH control forward 5′-GAAGTACACGGT

GGAGGCTG and reverse 5′-CGCTGATCACGACCTTCTTC primers. Complementary DNAs from intracellular *Leishmania* were synthesized from 40 ng of total RNA using the SuperscriptII™ RNase H⁻ Reverse Transcriptase (Invitrogen) and Oligo $(dT)_{12-18}$ primers (Invitrogen) following manufacturer's instructions. Real-time PCR was performed in triplicate in 25 μL volumes using IQ SybrGreen Super mix (Bio-Rad) for detection in a Rotor Gene-3000 (Corbett Research). Reactions were run using the following thermal profile: initial denaturation at 95°C for 4 min followed by 40 cycles with denaturation at 95°C for 20 s, annealing at 62°C for 20 s and extension at 72°C for 20 s. The PCR was followed by a melt curve analysis to ascertain that the expected products were amplified. The relative amount of PCR products generated from each primer set was determined based on the threshold cycle (Ct) value and amplification efficiencies, and was normalized by dividing the values by the relative amount of the *GAPDH* gene used as a control.

Thiol analysis

Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC) as described elsewhere.[10]

Western-blot analysis

Late log phase promastigotes (1×10^8) were harvested and the resultant cell pellet was resuspended in lysis buffer (20 mM MOPS, pH 7.2; 1 mM dithiothreitol; 2 mM phenylmethylsuphonylfluoride; 0.5 μg/mL each of leupeptin and aprotinin).

The cell pellet was lysed by sonication and cell supernatants were prepared by centrifugation at 20 000 **g**. Protein (50 μg) of promastigotes was fractionated by SDS–PAGE, blotted on to PVDF membranes using electrophoretic transfer cell (Bio-Rad) and probed with ODC antibody (diluted 1 : 3000, a gift from Dr Buddy Ullman, Oregon, USA). Western-blot analysis was done using the ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The results shown are from a single experiment typical of at least three giving identical results.

Statistical analysis

Data were statistically analysed by the Student's *t*-test. The data represent mean ± standard deviation (SD) of three determinations from at least two independent experiments. A P value of <0.05 was considered significant.

Results

Characterization of the resistance phenotype in clinical isolates

PFGE of the chromosomes of *L. donovani* clinical isolates derived from patients responding or not to standard SAG treatment indicated a similar karyotype, as no major differences were observed in chromosome number and sizes (results not shown). The sensitivity of these related clinical isolates to SAG was tested in intracellular amastigotes. The *Leishmania* expression vector (pGL-αNEOαLUC) containing the luciferase gene was transfected into the field isolates and the resulting transfectants were used for susceptibility assays (Figure 1). The IC_{50} values of AG83-S and 2001-S coming from SAG-responsive patients were 9 ± 0.5 μg/mL and 13 ± 1.5 μg/mL, respectively, whereas the field isolates 41-R, GE1-R, NS2-R and CK2-R coming from SAG-unresponsive patients had IC_{50}s of 65 ± 3.4 μg/mL, >100 μg/mL, 24 ± 1.4 μg/mL and 55 ± 2.5 μg/mL, respectively, as extrapolated from the predicted graphs.

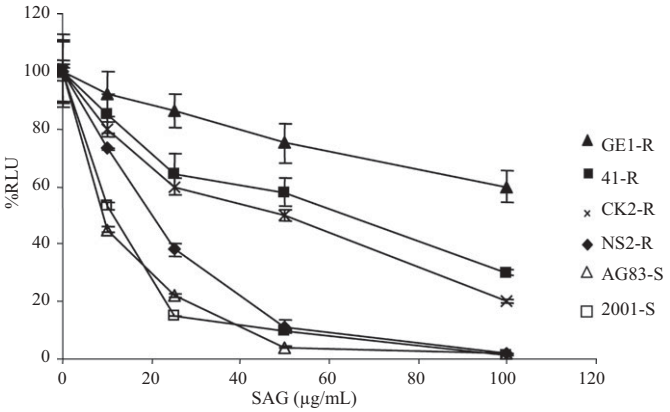

Figure 1. Susceptibility of sensitive (AG83-S and 2001-S) and resistant (41-R, GE1-R, NS2-R and CK2-R) field isolates to sodium antimony gluconate (SAG) as intracellular amastigotes. Intracellular survival of *Leishmania donovani* amastigotes, infecting J774A.1 macrophages in the presence of SAG. All the isolates were transfected with pGL-αNEOαLUC before infecting the macrophages as described in the Materials and Methods section. Each data point represents the mean ± SD of triplicates from three independent experiments.

Role of ABC transporter MRPA in antimony-resistant L. donovani clinical isolates

Gene amplification is a hallmark for drug resistance in *Leishmania*, at least when resistance is induced *in vitro*.[31–33] Gene amplification has also been noted in field isolates.[17,33] Previous reports on antimonial resistance have shown an increased expression of an ABC transporter MRPA.[8,29,34] To test whether *MRPA* was amplified in our field isolates, we prepared total DNA of field strains and hybridized the DNA to an *MRPA*-specific probe. Amplification of the *MRPA* gene was observed in three resistant isolates, GE1-R, NS2-R and CK2-R (Figure 2a). However, 41-R, another SAG-R isolate did not show any *MRPA* amplification (Figure 2a). The amplicons present in the resistant isolates were further analysed by PFGE. Amplicons found in the resistant isolates GE1-R, NS2-R and CK2-R were circular, as indicated by their characteristic migration in PFGE (Figure 2b) with the smears possibly corresponding to various topoisomers of the circles.[35] The circular nature of amplicons in resistant *Leishmania* isolates was further confirmed by isolating them by standard plasmid alkaline lysis preparation and migration in agarose gel. Comparison of DNAs derived from sensitive and resistant field isolates revealed circular amplified band in the resistant isolates GE1-R, NS2-R and CK2-R that were absent in the sensitive 2001-S and AG83-S and also in the 41-R isolates (Figure 3a). These circular amplicons from GE1-R, NS2-R and CK2-R were isolated and digested with *Hin*dIII. A similar digestion pattern was observed on the ethidium-bromide-stained gel (Figure 3b). Southern-blot analysis

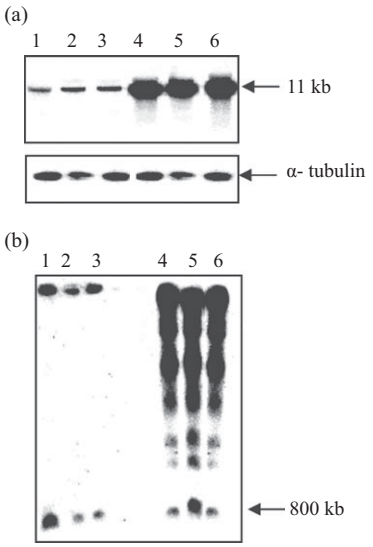

Figure 2. Amplification of *MRPA* gene in SAG-R isolates NS2-R and GE1-R. (a) Total genomic DNA of isolates was digested with *Hin*dIII, electrophoresed, blotted and hybridized with an *MRPA*-specific probe of 400 bp, derived from the *Leishmania tarentolae MRPA* gene. The sizes of the hybridizing bands were determined using *Hin*dIII-digested lambda DNA. The blot was rehybridized with an α-tubulin probe to monitor the amount of digested DNA layered on the gel. (b) CHEF blot hybridized with an *MRPA* probe. Chromosomes of SAG-S and SAG-R isolates were separated by PFGE and the Southern blot was hybridized with the same *MRPA* probe as above. The 800 kb chromosome showing the chromosomal localization of *MRPA* gene is present in all of the strains. Lane 1, AG83-S; lane 2, 2001-S; lane 3, 41-R; lane 4, GE1-R; lane 5, NS2-R; lane 6, CK2-R.

of the ethidium-bromide-stained gel with an MRPA-specific probe showed the presence of *MRPA* on the circular amplicon (Figure 3b). *MRPA* is part of the H locus, a region that also contains the pterin reductase gene (*PTR1*).[36,37] The *PTR1* gene was indeed found to be co-amplified with *MRPA* as determined by Southern-blot analysis (Figure 3b).

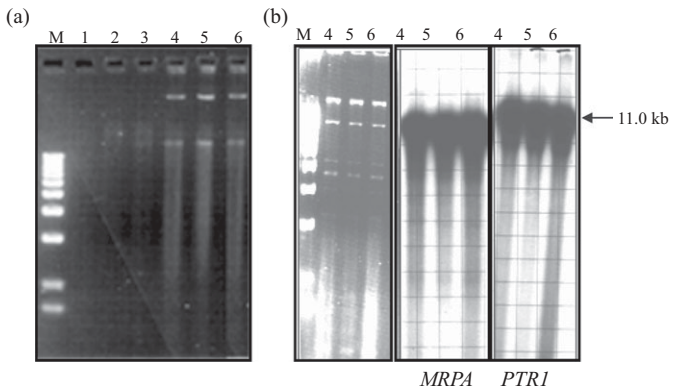

Figure 3. Circular DNA amplification and presence of *MRPA* and *PTR1* on the circles. (a) Ethidium-bromide-stained agarose gel showing circular DNA in SAG-R isolates. Alkaline lysed DNAs of SAG-S and SAG-R isolates were run on the agarose gel. (b) Isolated circles from GE1-R, NS2-R and CK2-R were digested with *Hin*dIII and the gel was stained with ethidium bromide. *Hin*dIII-digested circular DNA was hybridized with *MRPA*- and *PTR1*-specific probes. Lane 1, *Leishmania donovani* AG83-S; lane 2, 2001-S; lane 3, 41-R; lane 4, GE1-R; lane 5, NS2-R; lane 6, CK2-R.

Customized DNA microarrays containing all ABC protein genes of *Leishmania* were hybridized to GE1-R and 2001-S labelled cDNAs. MRPA was also found to be consistently overexpressed in GE1-R ($P < 0.01$) (A. Mukherjee, M. Ouellette and R. Madhubala, unpublished observations). Clinical resistance is found in the intracellular parasites while the molecular mutations described here were studied with the promastigotes. While it is unlikely that genes amplified in promastigotes would not lead to increase RNA in intracellular parasites, we carried out a real time RT-PCR assay of *MRPA* using intracellular amastigotes infecting the THP-1 cell line. An example of real time RT-PCR is shown in Figure 4(a). Increased expression of MRPA was indeed noted in GE1-R, NS2-R, and CK2-R but not in 41-R or the sensitive 2001-S lines (Figure 4b).

Role of γ-glutamylcysteine synthetase and ornithine decarboxylase in antimony resistance in the clinical isolates

In addition to *MRPA*, several loci have been reported to be amplified in antimony-resistant isolates, one being the *GSH1* gene coding for the heavy subunit of γGCS, the rate limiting enzyme for GSH synthesis.[12] Overexpression of ODC, the rate-limiting enzyme in polyamine biosynthesis, has also been reported in metal-resistant mutants.[11] The parasite-specific spermidine–GSH conjugate, trypanothione [$T(SH)_2$],[13] is known to bind to antimony, and it is proposed that the Sb-$T(SH)_2$ complex is either extruded outside the cell by an ATP-dependent efflux system[16] or sequestered within a vacuole by MRPA.[15] This prompted us to test whether *ODC* or *GSH1* was overexpressed in our field isolates resistant to SAG. Southern-blot analysis showed no gene amplification of *GSH1* (Figure 5a) although in the resistant strain 41-R, northern-blot hybridization with *GSH1* showed an increased expression of two transcripts of 2.4 and 3.4 kb in size when compared with the other isolates (Figure 5b). Southern-blot analysis of total genomic DNA digested with *Hin*dIII and hybridized with a full-length *ODC* probe showed an amplification of the *ODC* gene in 41-R, GE1-R, NS2-R and CK2-R as compared with the sensitive strains AG83-S and 2001-S (Figure 5c). Western-blot analysis further showed an increased expression of ODC protein (Figure 5d).

Thiol analysis in the clinical isolates

We also quantified thiol levels in the SAG-S and SAG-R isolates (Figure 6). Surprisingly, there were no differences in the $T(SH)_2$ levels between the SAG-S and SAG-R isolates (Figure 6c). However, the levels of cysteine were increased by 2-fold in the SAG-resistant strains GE1-R, NS2-R, and 2.8-fold in CK2-R when compared with the 2001-S isolate. The increase in cysteine level in 41-R was 3.5-fold when compared with 2001-S (Figure 6a). Similarly GSH levels showed 2.8-, 1.8-, 2.0- and 1.75-fold increases in 41-R, GE1-R, NS2-R and CK2-R, respectively, compared with 2001-S (Figure 6b). The levels of thiols were not significantly different between 2001-S and AG83-S.

Discussion

SAG is the drug of choice against *Leishmania* and resistance to this drug is a major problem in the field not only in the Indian subcontinent, but also throughout the world.[1,19–21,38–40] This increase in resistance to SAG has led to an upsurge in

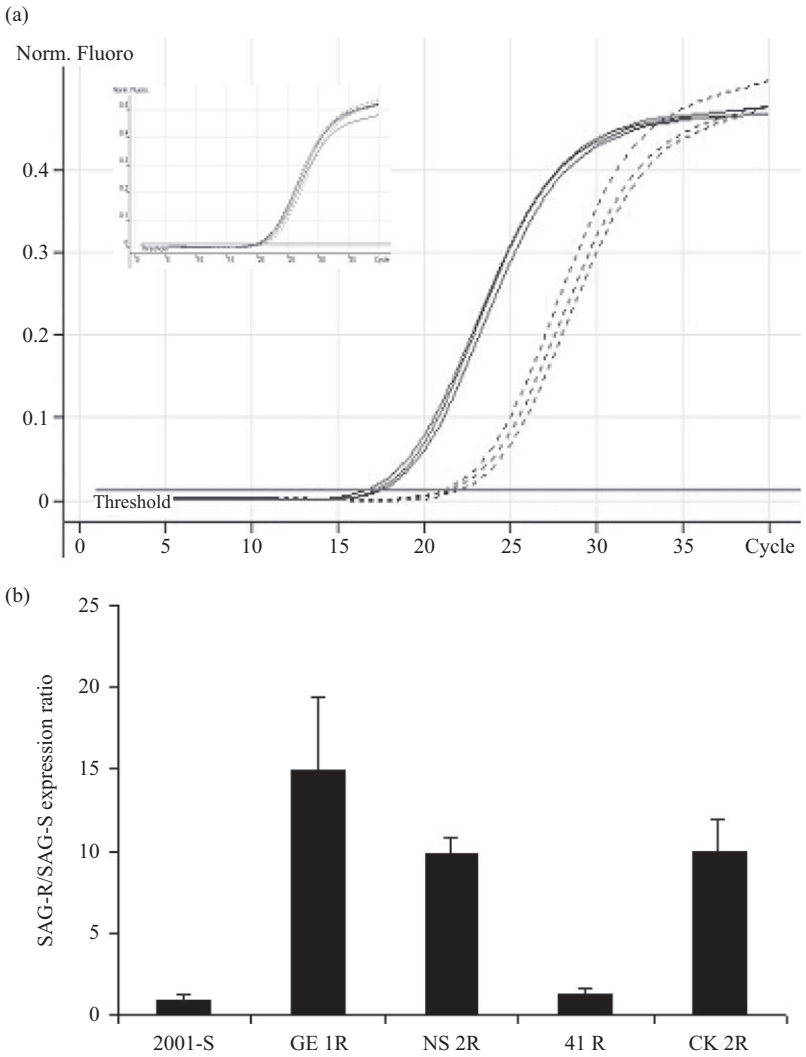

Figure 4. Real time RT-PCR expression analysis of *MRPA* in *Leishmania donovani* intracellular amastigotes. (a) Real-time RT-PCR fluorescence curves representing triplicates of the *MRPA* expression in intracellular amastigotes of 2001-S (dotted lines) and of GE1-R (continuous lines). The amplification curve of the *GAPDH* gene used for normalization is shown in the inset. (b) *MRPA* RNA expression ratios of sodium antimony stibogluconate (SAG)-resistant isolates relative to the SAG-sensitive isolate in intracellular amastigotes. Mean of three independent experiments performed from three different RNA preparations.

therapeutic failure, and with the limited chemotherapeutic alternatives, it is extremely relevant that mechanisms of resistance be evaluated in field isolates.[1]

Gene amplification, at least in strains made resistant under laboratory conditions, is a frequent mechanism by which *Leishmania* resists the action of cytotoxic drugs.[31–33,41] The first amplified gene in *Leishmania* promastigotes selected for arsenite resistance[34] or Sb(III) resistance[8] is the ABC transporter gene *MRPA*, which causes drug sequestration. Amplification of the ABC transporter gene *MRPA* has also been reported in an Sb(V) mutant of a *Leishmania guyanensis* cell line[42] or in axenic amastigotes of *Leishmania infantum* selected for resistance to Sb(III).[29] However, to date it remains an open-ended and unanswered question as to whether similar mechanisms exist in clinical isolates. To address this question we have characterized clinical isolates from India and report that linked but diverse mechanisms of resistance are operative in these isolates.

The ABC transporter MRPA has been shown to confer resistance to antimonials in promastigotes of *L. tarentolae* by sequestration of the metal thiol conjugates in an intracellular organelle located close to the flagellar pocket.[15] This model also appears to be applicable to the amastigotes.[29] For the first time we show that *MRPA* gene amplification is also taking place in some field isolates (Figure 2). MRPA is part of an extrachromosomal circle in GE-1-R, NS2-R, and CK2-R. This circular amplicon also encodes the pterin reductase gene (*PTR1*). Pterins are essential co-factors for *Leishmania* growth,[43,44] and co-amplification of *PTR1* may provide a growth advantage to resistant isolates under a number of conditions.

Previous studies using *in vitro* laboratory-generated drug-resistant *Leishmania* have shown that resistance to metals in *Leishmania* is multifactorial, with contributions by several independent mechanisms as mentioned earlier. In our study the *ODC* gene was amplified in the resistant isolates, 41-R, GE1-R, NS2-R and CK2-R. However, the *ODC* gene was not amplified on the extrachromosomal circles (data not shown). Western-blot analysis showed an increased expression of ODC protein in all the resistant isolates studied. Overexpression of *ODC* and *GSH1*

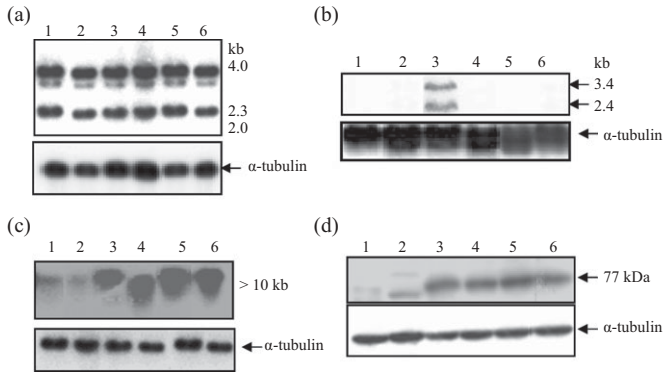

Figure 5. Characterization of γ-glutamylcysteine synthetase and ornithine decarboxylase in SAG-S and SAG-R isolates. (a) Genomic DNAs were isolated and digested with *Hin*dIII and hybridized to a *GSH1* probe. The sizes of the hybridizing bands were determined using *Hin*dIII-digested lambda DNA. The blot was rehybridized with an α-tubulin probe to monitor the amount of digested DNA layered on the gel. (b) The level of expression of *GSH1* gene was determined by northern-blot analysis of total RNA hybridized with a *GSH1* probe. An α-tubulin probe was used to monitor the amount of RNA layered on the gel. Sizes were derived from an RNA ladder. (c) Amplification of the *ODC* gene in *L. donovani* field isolates. Total genomic DNA of isolates was digested with *Hin*dIII, electrophoresed, blotted and hybridized with a full-length *ODC*-specific probe, derived from the *L. donovani ODC* gene. The blot was rehybridized with an α-tubulin probe to monitor the amount of digested DNA layered on the gel. (d) Overexpression of the ODC protein in the resistant isolates. Cell lysates of promastigotes were used for western-blot analysis for monitoring the expression of the 77 kDa ODC in the isolates. The same blot was reacted with antibody against α-tubulin protein to normalize the loading on to each lane of the gel. Lane 1, AG83-S; lane 2, 2001-S; lane 3, 41-R; lane 4, GE1-R; lane 5, NS2-R; lane 6, CK2-R.

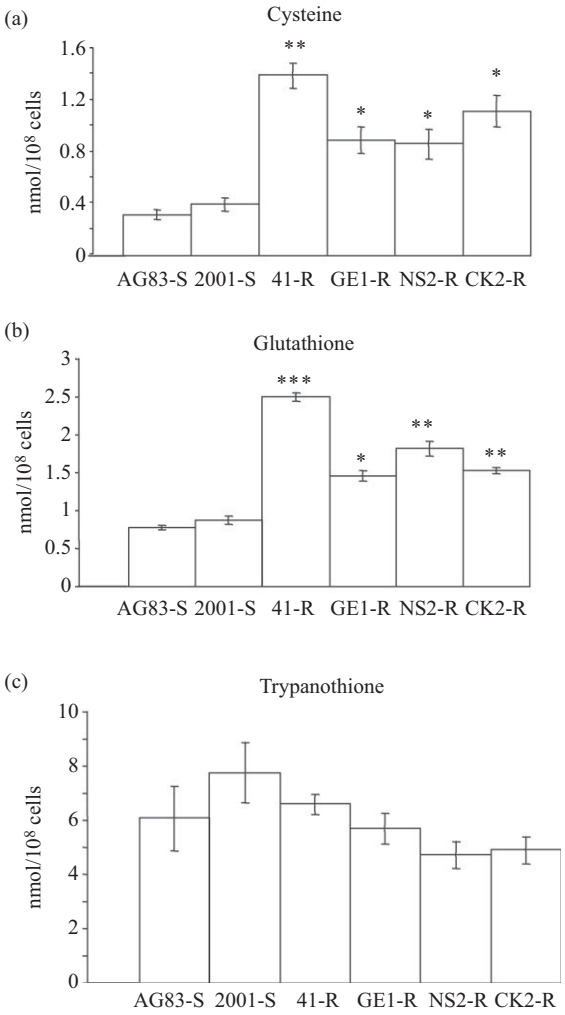

Figure 6. Intracellular levels of the thiols cysteine, glutathione (GSH) and trypanothione[$T(SH)_2$] in SAG-S and SAG-R clinical isolates. Thiols were derivatized with monobromobimane and separated by HPLC) Each value is a mean ± SD of triplicates from two independent experiments. (a) $*P \leq 0.04$–0.016; $**P = 0.006$. (b) $*P = 0.01$; $**P = 0.007$–0.005; $***P = 0.001$. (c) Not significantly different.

is usually linked to an increase, respectively, of polyamine or thiol levels, which can favour an increase in $T(SH)_2$ levels.[11] An increase in cysteine and GSH levels was observed (Figure 6) but we did not find any increase in trypanothione levels in any of the resistant isolates. This was also the case in *L. infantum* resistant to Sb(III).[29] This is possibly because the levels of $T(SH)_2$ in the *L. tarentolae* strain studied are much lower than in other cells. An alternative, non-exclusive, explanation is that the mode of action of Sb(V) was suggested to deplete $T(SH)_2$ by efflux of Sb-trypanothione conjugate.[14] Possibly this efflux system is increased in the resistant field strain, hence leading to increased trypanothione efflux and thus explaining a relatively constant level of trypanothione, despite that two biosynthetic steps are increased.

GSH1 was not amplified in any mutants but RNA overexpression was detected in 41-R. Overexpression without gene amplification has been observed in *Leishmania*-resistant cells[11] and it is possible that an increased RNA stability is responsible for the augmented levels of RNA. The increase in cysteine or GSH could possibly have a role in SAG resistance by binding either directly or indirectly to the metal compound. In our study we also observed that 41-R, the only strain that overexpressed *GSH1*, had much higher levels of cysteine and GSH when compared with GE1-R, NS2-R and CK2-R. It remains to be determined how cysteine and GSH are increased in field isolates that do not overexpress *GSH1*. It is possible that other thiol biosynthesis genes, as shown for *in vitro* resistant isolates,[25,29] are increased. However, the results of this study differ from a recent finding from studies on strains derived from Nepal,[30] raising the possibility that different resistance mechanisms are operating in neighbouring countries.

This is the first demonstration that resistance mechanisms found in laboratory strains can also be found in clinical kala azar *L. donovani* isolates. Increased thiols and MRPA overexpression are shown here to be also important resistance factors in the field. As clearly shown from the limited strains studied, the expression of a number of different genes appears to be implicated in SAG resistance. Further work is warranted in a larger number of isolates to test the frequency of the mutations detected and find possibly other resistance determinants.

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Transparency declarations

None to declare.

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