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Role of the ABC Transporter MRPA (PGPA) in Antimony Resistance in *Leishmania infantum* Axenic and Intracellular Amastigotes

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Antimonial compounds are the mainstay for the treatment of infections with the protozoan parasite *Leishmania*. We present our studies on *Leishmania infantum* amastigote parasites selected for resistance to potassium antimonyl tartrate [Sb(III)]. Inside macrophages, the Sb(III)-selected cells are cross-resistant to sodium stibogluconate (Pentostam), the main drug used against *Leishmania*. Putative alterations in the level of expression of more than 40 genes were compared between susceptible and resistant axenic amastigotes using customized DNA microarrays. The expression of three genes coding for the ABC transporter MRPA (PGPA), *S*-adenosylhomocysteine hydrolase, and folylpolyglutamate synthase was found to be consistently increased. The levels of cysteine were found to be increased in the mutant. Transfection of the *MRPA* gene was shown to confer sodium stibogluconate resistance in intracellular parasites. This MRPA-mediated resistance could be reverted by using the glutathione biosynthesis-specific inhibitor buthionine sulfoximine. These results highlight for the first time the role of MRPA in antimony resistance in the amastigote stage of the parasite and suggest a strategy for reversing resistance.

Leishmania is a protozoan parasite affecting several million people throughout the world. The clinical manifestations of the infection depend on the species, the most life-threatening being visceral leishmaniasis caused by the *Leishmania donovani* complex. Treatment relies exclusively on chemotherapy, and pentavalent antimonials [Sb(V)] are still the mainstay against all forms of *Leishmania* infections (14, 18). While Sb(V) is used for treating patients, it is generally agreed that Sb(V) is reduced to trivalent antimony [Sb(III)], which constitutes the active form of the drug against the parasite. The exact site of drug reduction (inside the macrophages or inside the parasites) is not known, but activities were recently discovered in *Leishmania* that could be implicated in this reduction process (7, 26, 29, 36). Resistance to Sb(V) is so widespread in part of India (33) that first-line treatment in this region is either based on miltefosine (31) or amphotericin B (32). Miltefosine is interesting because it can be taken orally, but single point mutations can lead to resistance (24), suggesting that resistance to this drug may occur rapidly.

Leishmania has a relatively simple life cycle with two main stages, the flagellated promastigote in the insect stage and the intracellular amastigote living inside macrophages. Progress in culture techniques has allowed the growth of *Leishmania* amastigotes as part of axenic cultures. An increase in the temperature from 25°C to 37°C and a decrease in the pH of the culture medium to mimic the conditions encountered in the phagolysosome are the key parameters to transform promastigotes into amastigotes (reviewed in reference 37). It is nonetheless easier to grow promastigotes, and most of the work pertaining to resistance mechanisms to antimonials was performed in the insect stage of the parasite. One resistance mechanism deduced from in vitro work involves multiple steps where antimony is conjugated to the parasite-specific glutathione-spermidine conjugate trypanothione (11). Trypanothione is thought to bind to metals in susceptible isolates (35) and is increased in Sb(III)-resistant cells (16). Metal-trypanothione conjugates are either sequestered into an intracellular organelle by the ABC transporter MRPA (formerly known as PGPA) (20) or extruded outside the cell by an efflux pump (8). It remains to be seen whether similar resistance mechanisms operate in the amastigote stage of the parasite and also in field isolates. Resistance in field isolates is less well defined, and it is only recently that susceptibility values determined using in vitro assays were found to correlate with the clinical response (21). Resistance in field isolates may involve amplification of genes (30). Resistance mechanisms in amastigotes have only recently been studied, and one resistance mechanism involves reduced accumulation of the drug (4). One possible route of entry of the drug is a parasite aquaglyceroporin (12). Another study has revealed a decrease in the conversion of Sb(V) to Sb(III) (29).

To further define the molecular mechanisms of resistance to antimonials in *Leishmania* amastigotes, we used customized DNA microarrays to screen for differentially expressed genes in an *L. infantum* axenic amastigote cell line selected for Sb(III) resistance. Our analysis indicates that the ABC transporter MRPA can confer resistance to antimony in intracellular amastigote parasites.

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MATERIALS AND METHODS

Cell lines. Wild-type clones of *L. infantum* (MHOM/MA/67/ITMAP-263) and *Leishmania viannia panamensis* strain MHOM/CO/86/1166 have been described

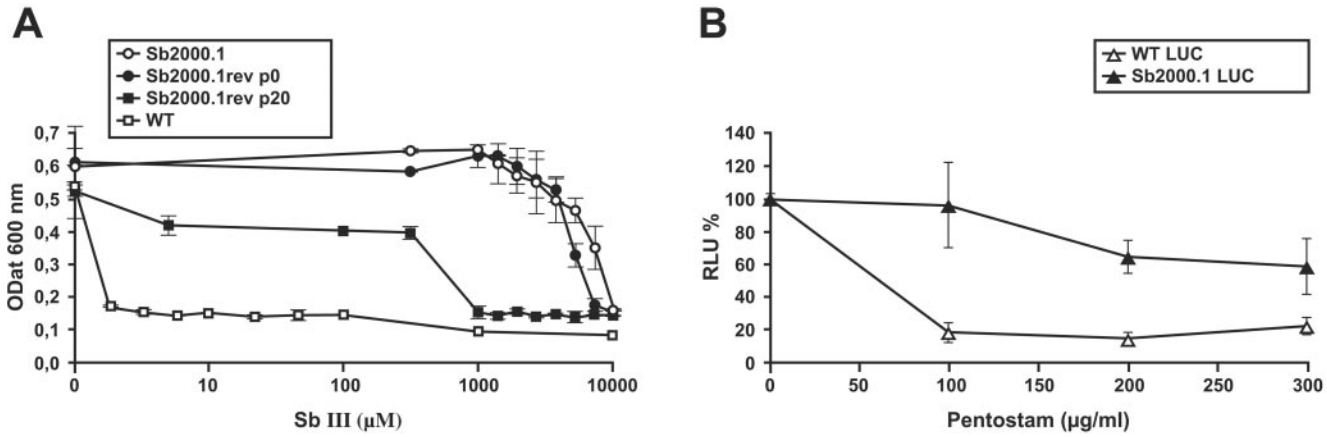

FIG. 1. Susceptibility of *L. infantum* amastigotes to antimonials. (A) Growth curve of *L. infantum* axenic amastigotes in the MAA medium in the presence of potassium antimonyl tartrate. (B) Intracellular survival of *L. infantum* axenic amastigotes infecting THP-1 cells in the presence of sodium stibogluconate. □, *L. infantum* wild type; ○, *L. infantum* Sb2000.1; ●, *L. infantum* Sb2000.1 grown for one passage in the absence of selection; ■, *L. infantum* Sb2000.1 grown for 20 passages in the absence of selection; Δ, *L. infantum* wild type transfected with 1.2 LUCαNEOα; ▲, *L. infantum* Sb2000.1 transfected with 1.2 LUCαNEOa.

previously (4, 28). Axenically grown amastigotes of *L. infantum* were maintained at 37°C with 5% CO_2 by weekly subpassages in MAA/20 (medium for axenically grown amastigotes) at pH 5.8 in 25-cm^2 flasks (27). The promastigote forms of *L. infantum* or *L. panamensis* were grown in the same medium at pH 7.0 at 25°C. Growth curves of control strains and transfectants in the presence of drugs were obtained by measuring absorbance at 600 nm using an automated microplate reader (Organon Teknica microwell system). An axenic *L. infantum* cell line was selected for Sb(III) resistance (potassium antimonyl tartrate hydrate; Aldrich Chemical) by step-wise selection starting with a drug concentration corresponding to the 50% effective concentration (EC_{50}) of the strain until we obtained the mutant *L. infantum* Sb2000.1, which was resistant to 2,000 μM. Note that the EC_{50} of susceptible axenic amastigotes varies from 2 to 20 μM, and this may depend on a number of factors. Nonetheless, the mutants are considerably more resistant than the sensitive isolates. This mutant was grown in the absence of drugs for 20 passages to obtain a (partial) revertant line.

DNA microarray procedures. We have recently described customized DNA microarrays for studying drug resistance in *Leishmania* (15). These arrays contain several genes implicated in drug resistance, including genes involved in cysteine, trypanothione, and folate metabolism and transport. These arrays were used to study gene expression in *L. infantum* Sb2000.1. RNA was isolated from mid-log-phase parasites with the TRIzol reagent (Invitrogen). The RNA was treated with DNase I (Ambion) and purified using the RNeasy kit (QIAGEN). RNA was converted to fluorescent cDNA probes by direct incorporation of Cy3/Cy5-linked dUTP (Amersham Pharmacia) mediated by the Superscript II reverse transcriptase (Invitrogen) according to the supplier's recommendations. For each labeling reaction mixture, 15 μg of purified RNA was spiked with two exogenous RNAs (*CAB1* at 2 pg/μl and *NAC1* at 5 pg/μl from *Arabidopsis thaliana*; Stratagene) to adjust for variations in the incorporation efficiency of the dyes and for differences in first-strand cDNA synthesis reactions. Aliquots of cDNA containing 20 pmol of incorporated dye were lyophilized and stored at −80°C until use. The hybridization, washes, data, and statistical analyses were done essentially as described previously using the QuantArray software (15). Statistical significance was calculated by Student's *t* test, using the StatView software. A cutoff of 2 for significant differences was chosen.

DNA constructs and transfections. MRPA constructs cloned into *Leishmania* expression vectors were described previously (20, 23). Briefly, the green fluorescent protein (GFP) was cloned in frame with MRPA, and this construct was subcloned into the *Leishmania* expression vector pSPY-NEO (20). These constructs were electroporated in *L. infantum* axenic amastigote cells and *L. panamensis* promastigotes as described elsewhere (28). The vector pSP1.2 LUC αHYGα (34) was also cotransfected in MRPA-transfected cells to facilitate the quantification of intracellular parasites.

Drug efficacy assay in THP-1 differentiated monocytes. The growth of the luciferase-expressing amastigotes of *L. infantum* and *L. panamensis* was evaluated in a human leukemia monocyte cell line (THP-1 cells) as described previously (28). Briefly, THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml. THP-1 cells in the log phase of growth were differentiated by incubation for 2 days in medium containing 20 ng of phorbol myristate acetate/ml (Sigma). These cells were washed with prewarmed medium and subsequently infected with stationary-phase *L. panamensis* promastigotes in 24-microwell plates (Falcon) at a parasite/macrophage ratio of 20:1 or with *L. infantum* axenic amastigotes at a parasite/macrophage ratio of 2:1. Noninternalized parasites were removed by several washes, and infected macrophages were put in contact with additive-free sodium stibogluconate (Pentostam). After 4 (for *L. panamensis*) or 5 (for *L. infantum*) days of drug exposure, wells containing adherent differentiated THP-1 cells were washed and the luciferase activity of the LUC-recombinant parasites was determined essentially as described elsewhere (25). Values were expressed as relative light units.

Confocal microscopy. *L. infantum* cells transfected with an MRPA-GFP construct were immobilized with 2% paraformaldehyde (Sigma) and mounted on microscope slides with coverslips. Samples were viewed with an Olympus confocal scanning laser system installed on an Olympus IX-70 inverted microscope with an argon laser. Visualization of the fluorophore was achieved using a 488-nm excitation filter and 510/530-nm emission filter. Samples were scanned for green fluorescence using a 60× objective (numerical aperture, 1.40) and a 5× zoom. Transmitted light images were collected at the same time as fluorescent images. Images were obtained using the Olympus Fluoview 300 software and processed using Adobe Photoshop software.

Thiols analysis. Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography as described elsewhere (22).

RESULTS

Characterization of the resistance phenotype in *L. infantum* Sb2000.1 axenic amastigotes. To study resistance to antimonials in the amastigote stage of the parasite, an *L. infantum* axenic amastigote line was selected for Sb(III) resistance (4). The EC_{50} of the wild-type sensitive line was close to 4 μM, while the Sb2000.1 line had an EC_{50} greater than 4,000 μM (Fig. 1A). The stability of the resistance phenotype was tested by passing the cells in the absence of Sb(III). Resistance was found to be stable, and it was only after 20 passages in the absence of the drug that part of the resistance present in the mutant was lost (Fig. 1A). This revertant axenic amastigote cell line had an EC_{50} of 800 μM, still 200-fold more resistant than the wild-type cell. These cells were grown for 20 more passages in the absence of drug, but the remaining resistance was stable (results not shown). In our cell culture assay, axenic *L. infantum* amastigotes are not intrinsically susceptible to pentavalent

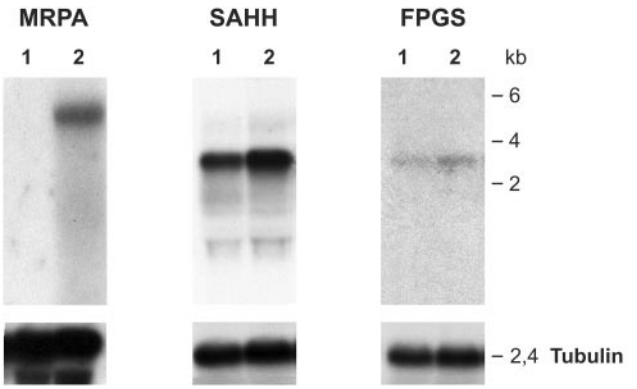

FIG. 2. Analysis of gene expression in *L. infantum* Sb2000.1 by Northern blot analysis. Total RNA from *L. infantum* amastigotes was hybridized to DNA probes recognizing the genes coding for the ABC transporter MRPA, SAHH, and FPGS. Hybridizations with a DNA probe covering the α-tubulin gene were done to monitor the amount of RNA layered. Sizes were derived from an RNA ladder. Lanes: 1, *L. infantum* wild type; 2, *L. infantum* Sb2000.1.

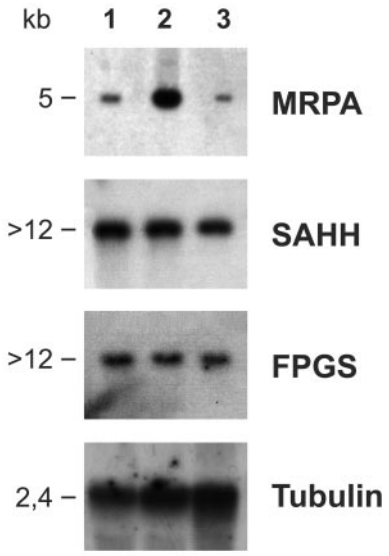

FIG. 3. Analysis of DNA copy number in *L. infantum* Sb2000.1 as determined by Southern blot analysis. The DNAs of the parasites were isolated and digested with XhoI for the blots hybridized to the *MRPA* probe and with EcoRI for the blots hybridized with the *SAHH* and *FPGS* probes. DNA loading was monitored by ethidium bromide staining, and the filter hybridized with the MRPA probe was rehybridized with an α-tubulin probe. Lanes: 1, *L. infantum* wild type; 2, *L. infantum* Sb2000.1; 3, *L. infantum* Sb2000.1 revertant grown in the absence of drug for 20 passages. The sizes of the hybridizing bands were determined using a DNA 1-kb plus ladder.

antimony [Sb(V)] (EC_{50} of 1,250 μg/ml), but they are susceptible to sodium stibogluconate when infecting macrophages, with an EC_{50} of 75 μg/ml (Fig. 1B). Interestingly, the Sb2000.1 mutant selected for Sb(III) resistance was cross-resistant to sodium stibogluconate in our macrophage intracellular assay (Fig. 1B).

Analysis of resistance mechanisms in *L. infantum* by DNA microarrays. For analyzing the expression of several drug resistance genes in parallel, we have made customized DNA microarrays with PCR fragments containing 44 genes involved in cysteine, glutathione, trypanothione, folate, pterin, and methionine biosynthesis and transport (15). These arrays have already shown their usefulness for the study of drug resistance in various *Leishmania* species (15). We hypothesized that these arrays would be useful to study resistance in the amastigote stage of the parasite. RNAs, derived from *L. infantum* Sb2000.1 and the susceptible parent strain, were reverse transcribed, labeled, and hybridized to the customized DNA microarrays. From the selected genes covered by the array, none was found to be downregulated in the mutant, but three genes appeared to be consistently upregulated as determined by DNA microarrays when we applied a cutoff of at least twofold differential expression. One gene corresponds to the ABC transporter MRPA (PGPA) ($P < 0.003$), a second one to *S*-adenosylhomocysteine hydrolase (SAHH) ($P < 0.01$), and the third one to folylpolyglutamate synthase (FPGS) ($P < 0.0001$). These microarray results were further studied by Northern blot analysis, and all three genes were indeed found to be expressed at higher levels in the resistant mutant than in susceptible cells (Fig. 2). Under our experimental conditions, the RNA corresponding to MRPA could not be detected in wild-type cells. Increased expression in *Leishmania*, at least in the promastigote stage, is sometimes the result of gene amplification (2, 3), but clearly other mechanisms can also lead to an increase in RNA (9, 17). The DNAs derived from *L. infantum* wild-type and Sb2000.1 cells were isolated, digested, blotted, and hybridized to MRPA, SAHH, and FPGS probes. The *MRPA* gene was found to be amplified (Fig. 3A, lane 2). Additional work has shown that *MRPA* is part of an extrachromosomal circle (results not shown). This amplified DNA was lost in cells passaged 20 times in the absence of the drug (Fig. 3A, lane 3). Concomitant with the loss of this amplicon, part of the resistance was also lost in the revertant line (Fig. 1A). Neither the *SAHH* gene nor the *FPGS* gene was amplified (Fig. 3), however.

Role of the ABC transporter MRPA in antimony resistance in *Leishmania* amastigote parasites. In *Leishmania tarentolae* promastigotes, MRPA is located intracellularly and has been proposed to confer resistance by sequestering the antimonials conjugated to thiols into an intracellular organelle (20). We first tested whether transfection of MRPA in *L. infantum* axenic amastigotes could lead to Sb(III) resistance in this stage of the parasite. Growth curve experiments clearly showed that MRPA can indeed bestow resistance to Sb(III) in axenic amastigotes (Fig. 4A). We also fused GFP to the MRPA C terminus to yield MRPA-GFP. The MRPA-GFP fusion was active, as it led to a twofold increase in resistance to Sb(III) in axenic amastigotes, a level also observed with transfectants with the unfused MRPA (Fig. 4A). The increase in resistance parallels that observed in promastigotes (20). The location of the MRPA-GFP fusion was studied by confocal microscopy in axenic amastigotes and was also found to be located intracellularly in this stage of the parasite (Fig. 4B).

MRPA is thought to transport the antimonials conjugated to thiols, including trypanothione, and, consistent with this hypothesis, trypanothione and other thiols were found to be increased in a number of resistant mutants of different *Leishmania* species (16, 19, 22). Thiol levels were measured in the

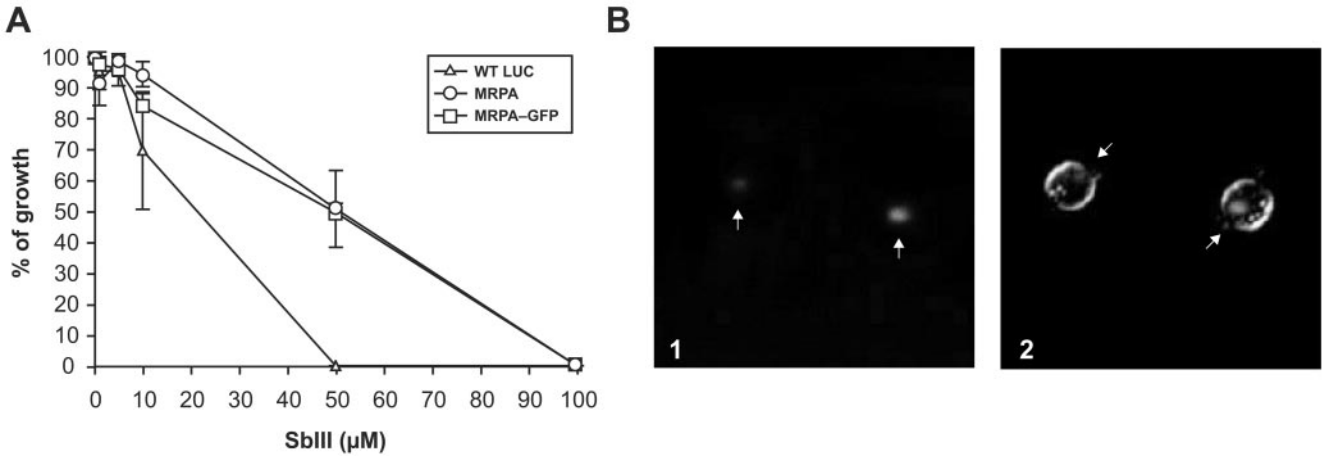

FIG. 4. MRPA is an intracellular ABC transporter that confers antimony resistance in *L. infantum* axenic amastigotes. (A) Transfection of *MRPA* in *L. infantum* axenic amastigotes leads to Sb(III) resistance. Δ, *L. infantum* transfected with the vector control 1.2LUCαNEOα, compared with *L. infantum* transfected with constructs containing MRPA (○) or MRPA-GFP (□). (B) Localization of MRPA-GFP was studied by confocal microscopy in *L. infantum* axenic amastigotes. Lanes: 1, fluorescence only; 2, composite image of Nomarsky differential interference contrast and fluorescence.

susceptible and resistant axenic amastigote culture of *L. infantum*. While glutathione and trypanothione levels were similar between the wild-type and Sb2000.1-resistant cells, the levels of cysteine were increased by twofold in the mutant compared to the susceptible cells (Fig. 5).

MRPA confers resistance to Sb(III) in axenic amastigotes (Fig. 4A) and, while Sb(V) is likely to be converted to Sb(III) (7, 26, 36), Sb(V) is nonetheless the drug used in the treatment of *Leishmania* infections. Sb(V) has very little activity against our axenic amastigote line (27), and we therefore decided to test the role of MRPA in intracellular parasites. Attempts to study MRPA using *L. infantum* have been inconclusive, in part because of the rather high intrinsic resistance of the susceptible strain (Fig. 1 and unpublished observations). In contrast, *L. panamensis* was ~10 times more sensitive to Sb(V) in our assay (Fig. 6A), and we thus transfected the *MRPA* construct in *L. panamensis*. These recombinant parasites were used for infecting the human monocyte cell line THP-1. Most interestingly, the MRPA transfectants were clearly more resistant to Sb(V) than were the control transfected lines (Fig. 6A). Buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthase, the rate-limiting step in glutathione biosynthesis, was shown to reduce antimony resistance in vitro (17) and in vivo (5). We tested whether it could reduce MRPA-mediated resistance in intracellular parasites. The results indicated that while the susceptibility of control transfectants was not altered (Fig. 6C), the resistance mediated by MRPA in intramacrophagic parasites was abrogated in the presence of BSO (Fig. 6B).

DISCUSSION

Resistance to antimony has been induced in axenic amastigotes (4, 27, 29), although no genes have so far been linked to resistance. In one study, a decrease in the rate of reduction of Sb(V) to Sb(III) was correlated to Sb(V) resistance (29). A number of *Leishmania* proteins have recently been discovered that could be related to this reductase activity (7, 36), although the molecular defects in the resistant line remains to be discovered. Transport defects were also observed in *L. infantum* axenic resistant mutants (4), and while one route of entry for

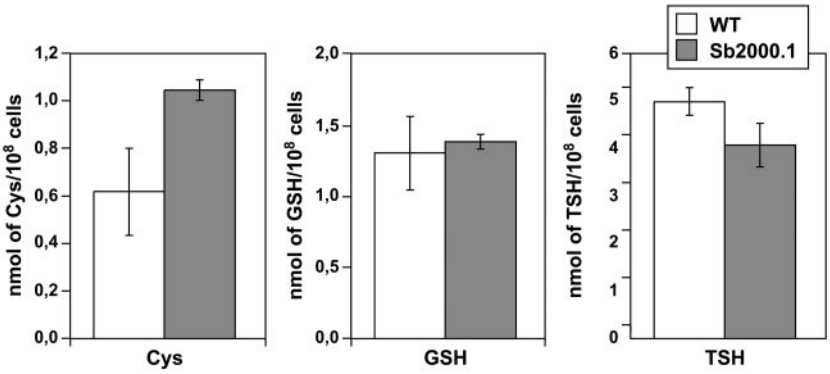

FIG. 5. Quantification of intracellular cysteine, glutathione, and trypanothione. Thiols of cells were prepared as described in Materials and Methods and analyzed by high-performance liquid chromatography. Averages of triplicates are shown; the experiment was repeated once with essentially the same results.

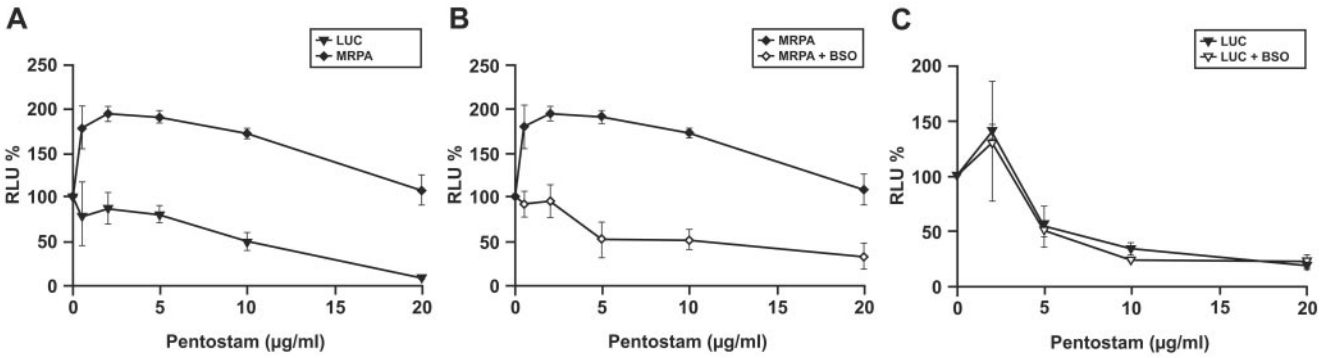

FIG. 6. MRPA-mediated resistance in intracellular parasites and reversal using BSO. (A) *L. panamensis* strains transfected with MRPA (♦) and infecting macrophages are resistant to sodium stibogluconate, compared to transfected control cells (▼). (B) The glutathione-specific inhibitor BSO abrogated the resistance mediated by MRPA in intracellular parasites (◇). (C) Susceptibility of control *L. panamensis* cells transfected with pSP72 αneoα and 1.2 LUC HYG was not altered in the presence of BSO (▽).

Sb(III) in *Leishmania* implicates the aquaglyceroporin AQP1 (12), we have no evidence, at least at the DNA level, that AQP1 is altered in Sb2000.1 (unpublished observations). DNA microarrays have recently been used to study drug resistance in microorganisms (1, 6), including *Leishmania* (15). Using DNA microarrays containing a limited number of genes, we found three genes whose expression levels were consistently altered in the Sb2000.1 resistant mutant. One gene consists of *MRPA*, and the role of MRPA was confirmed by gene transfection in axenic amastigotes (Fig. 4) but even more importantly in intracellular parasites (Fig. 6A). MRPA was shown to confer resistance to antimonials in promastigote cells by sequestration of the metal-thiol conjugates in an intracellular organelle located close to the flagellar pocket (20). This model also appears to apply to amastigotes, since MRPA is an intracellular protein, consistent with a localization close to the flagellar pocket (Fig. 4B). Clearly, resistance genes other than *MRPA* are present in the drug-selected mutant, since the loss of the *MRPA*-containing amplicon in the revertant is associated with a modest loss in resistance but the revertant cell is still considerably more resistant than the wild-type susceptible isolate (Fig. 1A). MRPA, being an intracellular protein, is likely not responsible for the defect in antimonial accumulation described in axenic amastigote resistant mutants (4). At least two transport systems are therefore involved in resistance: one corresponds to MRPA, and a second one corresponds either to the aquaglyceroporin AQP1 (12) and/or a previously described efflux system (8) that still needs to be identified.

Two other genes were found to be overexpressed, *SAHH* and *FPGS*. SAHH is involved in the conversion of *S*-adenosyl homocysteine to homocysteine, a cysteine precursor. Interestingly, we found that cysteine levels were significantly higher in the mutant (Fig. 5). Possibly, an increase in cysteine can be either directly or indirectly (e.g., by serving as a precursor for glutathione in trypanothione biosynthesis) involved in the resistance phenotype by reacting with metals. These metal-thiol conjugates would be substrates for transporters. The *SAHH* gene was found to be amplified also in an *L. tarentolae* mutant selected for Sb(III) resistance (15) although, while the *SAHH* gene was overexpressed in Sb2000.1, the gene was clearly not amplified (Fig. 3C). FPGS is involved in the addition of glutamic acid residues to folic acid, and this gene is linked to methotrexate resistance (10). It is not clear how FPGS would be involved in Sb(III) resistance, but it is expected that a number of changes may occur in a cell upon drug selection (9) and some of these changes may not be directly linked to the resistance phenotype studied. These expression results confirm, however, that there are several mechanisms outside gene amplification that can lead to RNA overexpression despite the fact that this protozoan parasite is devoid of classical RNA polymerase II promoters.

In our study of antimony-resistant *L. tarentolae*, cysteine, glutathione and, in particular, trypanothione were found to be increased (16, 22). Cotransfection of glutathione biosynthesis genes and MRPA were shown to be synergistic when in the right background (13, 20). In *L. infantum*, the baseline levels of trypanothione are much higher (50-fold) than in *L. tarentolae* (5 versus 0.1 nmol/10^8 cells). An increase in trypanothione levels may therefore not be as essential in *L. infantum* for achieving resistance to antimonials, although it is worth pointing out that cysteine is increased. Thiols are nonetheless required for MRPA-mediated resistance, since treatment with BSO, a known specific inhibitor of glutathione biosynthesis, reversed MRPA-mediated resistance inside macrophages (Fig. 6B). Treatment with BSO was shown previously to reduce resistance to antimonials not only in vitro (17) but also in vivo (5). It is possible that BSO affects the thiol levels of both the macrophage and the parasite, and the collective effect would be decreased resistance to Sb(V) mediated by MRPA.

A major finding of this study is the ability of MRPA to confer Sb(V) resistance inside macrophages. The increase in resistance is quite impressive (Fig. 6) when taking into account that MRPA has a relatively modest effect in axenic amastigotes (Fig. 4A) or in promastigotes (23). It is possible that resistance is better achieved in the intracellular stage of the parasite or that the exquisite sensitivity of *L. panamensis* to Sb(V) makes it an ideal model. The resistance to Sb(V) mediated by MRPA (Fig. 6A) is similar to the increase in resistance observed with Sb2000.1 (Fig. 1), despite these cells being much more resistant to Sb(III) than MRPA transfectants. It is thus possible that the intracellular assay may not be quantitative and may only provide qualitative information on whether a strain is sensitive or resistant. Nonetheless, the intracellular assay has shown that it can correlate well with the clinical responses (21). Thus, if

MRPA-overexpressing strains are present in the field, our results would suggest that these will be resistant to Sb(V). One possible way to circumvent resistance would be to provide a combination therapy consisting of Sb(V) and BSO, a combination that has been shown useful in animal models on field isolates with unknown genetic background (5).

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