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The LABCG2 Transporter from the Protozoan Parasite *Leishmania* Is Involved in Antimony Resistance

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Treatment for leishmaniasis, which is caused by *Leishmania* protozoan parasites, currently relies on a reduced arsenal of drugs. However, the significant increase in the incidence of drug therapeutic failure and the growing resistance to first-line drugs like antimonials in some areas of Northern India and Nepal limit the control of this parasitic disease. Understanding the molecular mechanisms of resistance in *Leishmania* is now a matter of urgency to optimize drugs used and to identify novel drug targets to block or reverse resistant mechanisms. Some members of the family of ATP-binding cassette (ABC) transporters in *Leishmania* have been associated with drug resistance. In this study, we have focused our interest to characterize LABCG2's involvement in drug resistance in *Leishmania*. *Leishmania major* parasites overexpressing the ABC protein transporter LABCG2 were generated in order to assess how LABCG2 is involved in drug resistance. Assays of susceptibility to different leishmanicidal agents were carried out. Analysis of the drug resistance profile revealed that *Leishmania* parasites overexpressing LABCG2 were resistant to antimony, as they demonstrated a reduced accumulation of Sb^{III} due to an increase in drug efflux. Additionally, LABCG2 was able to transport thiols in the presence of Sb^{III}. Biotinylation assays using parasites expressing LABCG2 fused with an N-terminal green fluorescent protein tag revealed that LABCG2 is partially localized in the plasma membrane; this supports data from previous studies which suggested that LABCG2 is localized in intracellular vesicles that fuse with the plasma membrane during exocytosis. In conclusion, *Leishmania* LABCG2 probably confers antimony resistance by sequestering metal-thiol conjugates within vesicles and through further exocytosis by means of the parasite's flagellar pocket.

Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoan parasites and is spread by the bite of infected phlebotomine sand flies. Currently, 1.3 million new cases of leishmaniasis and 20,000 to 30,000 deaths occur annually through a variety of clinical presentations (1).

Although chemotherapy is the only current treatment option for leishmaniasis, its efficacy is increasingly limited by growing resistance to first-line drugs, especially antimonials; the frequent side effects associated with their use; and the high cost of treatment. There is a limited number of drugs available for treatment, including amphotericin B, especially as a liposomal formulation; paromomycin; miltefosine; or pentavalent antimonials. The World Health Organization recently recommended the use of a combination of leishmanicidal drugs in order to decrease the concentration and toxicity of the dosages required as well as to delay the development of resistance. Even so, emerging drug resistance constitutes one of the main problems facing current leishmaniasis chemotherapies. In India, 60% of patients suffering from visceral leishmaniasis do not respond to treatment with antimonials due to the parasite's increased resistance to these drugs (2).

One of the most characteristic mechanisms of antimony resistance in *Leishmania* is drug efflux mediated by ABC (ATP-binding cassette) transporters such as MRPA (formerly PGPA)/ABCC3 (3, 4) or ABCI4 (5), which results in a reduced degree of antimony accumulation in parasites. ABC transporters are one of the largest protein families known; they are highly evolutionarily conserved from bacteria to humans and are involved in the transport of different compounds through biological membranes. *Leishmania* has 42 ABC genes distributed across nine subfamilies (ABCA to ABCI), yet to date, only some transporters found in the ABCA, ABCB, ABCC, ABCG, and ABCI subfamilies have been characterized.

Overexpression of MRPA and ABCI4 in *Leishmania* confers Sb^{III} resistance to the promastigote forms and Sb^{III} or Sb^{V} resistance to the intracellular amastigote forms (5, 6). Leishmaniasis is treated with antimonials by using pentavalent antimony-based drugs. Sb^{V} can be taken up by amastigotes and reduced to Sb^{III} inside macrophages so that it may become active against *Leishmania* parasites. This mechanism has not been fully elucidated, and there is apparently more than one Sb^{V}-to-Sb^{III} conversion route. Reduced glutathione (GSH) has been observed to promote the reduction of Sb^{V} to Sb^{III} in the phagolysosomes of macrophages (7). Alternatively, parasite-specific thiol-dependent reductase 1 (TDR1) and arsenate reductase (ACR2) found in *Leishmania* are also able to reduce Sb^{V} to Sb^{III} (8, 9). As described previously for MRPA, the resulting Sb^{III} can combine with thiols to form conjugates inside intracellular organelles, which are then effluxed from the parasite (3).

The involvement of the LABCG2 transporter in the phosphatidylserine (PS) externalization required for host macrophage infection was reported previously (10). Although PS synthesis in *Leishmania* has been a matter of intense debate, it was concluded that parasites in the late logarithmic phase contain PS (11, 12).

In *Leishmania*, LABCG4 and LABCG6 transporters have been

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involved in phosphatidylcholine transport and also confer resistance to different drugs, including miltefosine (13, 14). Considering that some ABC transporters present pleiotropic activity in response to therapeutic xenobiotics, we focused on the role of the *Leishmania* LABCG2 transporter in drug resistance.

MATERIALS AND METHODS

Chemical compounds. Trivalent antimony (Sb^{III}) (potassium antimony tartrate), trivalent arsenite (As^{III}) (sodium meta-arsenite), amphotericin B, pentamidine, chloroquine, quinine, mefloquine, primaquine, vinblastine, G-418 (Geneticin), buthionine-(*S*,*R*)-sulfoximine (BSO), 4′,6-diamidino-2-phenylindole dilactate (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *n*-dodecyl-β-D-maltopyranoside (DDM), $CdCl_2$, $CoCl_2$, $CuSO_4$, and GSH were purchased from Sigma-Aldrich. Miltefosine and perifosine were purchased from ÆternaZentaris. Pentavalent antimony (Sb^V) (sodium stibogluconate), tafenoquine, and sitamaquine dihydrochloride were provided by GlaxoSmithKline. Daunomycin was purchased from Pfizer.

***Leishmania* culture conditions.** Promastigotes of *Leishmania major* (MHOM/JL/80/Friedlin) and derivative lines used in this study were cultured at 28°C in RPMI 1640-modified medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (hiFBS; Invitrogen).

Gene expression. Total RNA from different *L. major* lines was extracted by using the High Pure RNA isolation kit (Roche Diagnostics GmbH). RNA was transcribed into cDNA by employing the qScript cDNA synthesis kit (Quanta Biosciences, Inc.) according to the manufacturer's instructions. The cDNA obtained was diluted (1:10 and 1:50), amplified with sense (5′-CCTACAGAGGACACCTACA) and antisense (5′-GAAGGGATTCTGGCAAG) primers for *LABCG2* and with sense (5′-GAAGTACACGGTGGAGGCTG) and antisense (5′-CGCTGATCAC GACCTTCTTC) primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (as an internal control), and electrophoresed on a 4% agarose gel.

Cell transfection and susceptibility analysis. Promastigotes of *L. major* were transfected with the previously described pUCNEO (empty vector), pUCNEO-*LABCG2*, and pXG-*GFP*::*LABCG2* constructs (10) and selected for G-418 resistance, as described previously (15). The susceptibilities of the respective pUCNEO (control), LABCG2, and GFP-LABCG2 promastigote lines to different compounds were determined by using an MTT colorimetric assay, as described previously (16). To analyze the relationship between thiol levels and susceptibility to Sb^{III}, parasites were previously grown in M199 culture medium supplemented with 10% hiFBS plus 3 mM BSO (a γ-glutamylcysteine synthetase inhibitor) for 48 h at 28°C. For assays of susceptibility of intracellular *Leishmania* amastigotes to Sb^{III} and Sb^V, stationary-phase promastigotes were used to infect macrophage-differentiated THP-1 cells at a macrophage/parasite ratio of 1:10, as described previously (17). After overnight infection at 35°C with 5% CO_2 in RPMI 1640 medium plus 5% hiFBS, extracellular parasites were removed by washing with serum-free medium. Infected macrophages were incubated at 37°C with different concentrations of Sb^{III} and Sb^V with 5% CO_2 in RPMI 1640 medium plus 10% hiFBS for 72 and 120 h, respectively. Following incubation, the cultures were fixed and analyzed as described previously (17).

Cell surface biotinylation. Parasite (1×10^8 promastigotes) surfaces were labeled as described previously (18) but by using 3% DDM instead of 1% Nonidet P-40 to cause parasite lysis and with a 60-min incubation instead of a 30-min incubation in lysis buffer coupled with a protease inhibitor cocktail (Sigma-Aldrich). Protein samples were fractionated by SDS-PAGE under standard conditions and electrotransferred onto Immobilon-P membranes (Millipore). Immunodetection was performed by using a 1:5,000 dilution of polyclonal anti-green fluorescent protein (GFP) (Rockland Immunochemicals) or a 1:3,000 dilution of polyclonal anti-plasma membrane (PM) protein-LRos3 (18) in phosphate-buffered saline (PBS) plus 0.01% Tween 20 and 0.1% bovine serum albumin (BSA). Control over PM integrity was determined by immunodetection using a monoclonal anti-cytosolic tryparedoxin peroxidase antibody at a 1:6,000 dilution (a gift from Ana M. Tomás, IBMC, Porto, Portugal). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit (1:5,000) immunoglobulin G (Dako) for GFP, LRos3, and tryparedoxin peroxidase. Signals were detected by using the ECL chemiluminescent substrate (Pierce).

Antimony accumulation and efflux. Promastigotes (10^8/ml) were incubated at 28°C with 100 μM Sb^{III} in RPMI 1640 culture medium at 28°C for different times. The parasites were centrifuged and pelleted to measure antimony accumulation after each time period (19). Antimony efflux was determined by incubating the different promastigote lines with compensated Sb^{III} concentrations (100 μM for the pUCNEO line and 200 μM for the LABCG2 line) for 1 h at 28°C in culture medium in order to attain similar labeling in *Leishmania* lines. The parasites were then washed with PBS and resuspended in culture medium at 28°C, and the pellet was collected after different time points. The samples for uptake and efflux determination were measured by using inductively coupled plasma mass spectrometry (ICP-MS; PerkinElmer) as described previously (5).

Determination of nonprotein thiol levels. Log-phase parasites (10^7 parasites/ml) were grown in M199 medium plus 10% hiFBS in order to measure thiol levels. They were then washed with PBS and incubated at 37°C with 2 μM CellTracker for 15 min. After incubation, the parasites were again washed with PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson). Fluorescence emission was quantified at between 515 and 545 nm by using Cell Quest software. The efflux of nonprotein thiols to the culture medium was determined by using the ThioStar thiol fluorescent detection reagent (Luminos) as described previously (5).

TABLE 1 Drug resistance profile in promastigote *L. major* lines[a]

Drug	Mean EC_{50} (μM) ± SD (RI)[b]		
	pUCNEO	LABCG2	LABCG2rev 90D
Sb^{III}	**16.02 ± 2.63**	**118.84 ± 11.50 (7.4)***	**11.53 ± 0.69 (0.7)**
As^{III}	**0.99 ± 0.30**	**6.02 ± 1.50 (6.0)***	**1.32 ± 0.02 (1.3)**
Amphotericin B	2.27 ± 0.73	2.33 ± 0.45 (1.0)	—
Miltefosine	18.25 ± 0.22	17.64 ± 1.70 (0.9)	—
Pentamidine	**0.66 ± 0.11**	**1.03 ± 0.11 (1.6)***	**0.83 ± 0.03 (1.3)**
Tafenoquine	12.87 ± 3.16	15.92 ± 7.50 (1.2)	—
Sitamaquine	21.88 ± 5.43	18.02 ± 0.82 (0.8)	—
Primaquine	5.43 ± 0.32	6.11 ± 0.05 (1.1)	—
Chloroquine	10.99 ± 0.53	9.37 ± 1.67 (0.8)	—
Daunomycin	**0.56 ± 0.09**	**1.06 ± 0.16 (1.9)***	**0.67 ± 0.06 (1.2)**
Mefloquine	2.02 ± 0.19	3.04 ± 0.34 (1.5)	—
Quinine	23.62 ± 2.84	29.69 ± 0.26 (1.3)	—
Perifosine	20.99 ± 1.95	20.96 ± 1.27 (1.0)	—
Vinblastine	10.31 ± 2.16	13.25 ± 2.65 (1.3)	—

[a] Promastigotes of *Leishmania* lines were grown for 72 h at 28°C in the presence of increasing concentrations of drugs. Cell viability was determined by using an MTT-based assay as described in Materials and Methods. Bold font represents significant resistance.

[b] Resistance indexes (RI) were calculated by dividing the EC_{50} for the *Leishmania* line overexpressing LABCG2 and LABCG2rev 90D by that for the *Leishmania* control line (pUCNEO). Data are the means ± standard deviations of results from three independent experiments. Significant differences were determined by using the Student *t* test (*, $P < 0.01$). —, not determined.

RESULTS AND DISCUSSION

Overexpression of LABCG2 confers resistance to antimony and other compounds. The role of ABC transporters in resistance to different compounds has been studied previously (5, 19–21). As mentioned above, the *Leishmania* LABCG2 transporter is involved in the PS externalization required for macrophage infection (10). We have determined that the overexpression of LABCG2 in *Leishmania*

TABLE 2 Heavy metal resistance profiles of *L. major* promastigote lines[a]

Metal	Mean EC_{50} (μM) ± SD (RI)[b]			
	pUCNEO	LABCG2	2-LABCG2	GFP-LABCG2
Sb^{III}	**16.02 ± 2.63**	**118.84 ± 11.50 (7.4)***	**42.92 ± 7.75 (2.7)***	**34.93 ± 1.00 (2.2)***
As^{III}	**0.99 ± 0.30**	**6.02 ± 1.50 (6.0)***	**2.72 ± 0.78 (2.7)***	**3.20 ± 0.18 (3.2)***
Cd^{II}	58.77 ± 0.82	52.79 ± 4.21 (0.9)	—	—
Co^{II}	28.46 ± 4.05	36.57 ± 5.27 (1.3)	—	—
Cu^{II}	43.52 ± 7.02	60.68 ± 4.99 (1.4)	—	—

[a] Promastigotes of *Leishmania* lines were grown for 72 h at 28°C in the presence of increasing concentrations of metals. Cell viability was determined by using an MTT-based assay as described in Materials and Methods. Bold font represents significant resistance.

[b] Resistance indexes (RI) were calculated by dividing the EC_{50} for *Leishmania* line overexpressing LABCG2, LABCG2rev at 90 days (LABCG2rev 90D), second-event LABCG2 (2-LABCG2), and GFP-LABCG2 by that for *Leishmania* control line (pUCNEO). Data are the means ± standard deviations of results from three independent experiments. Significant differences were determined by using the Student *t* test (*, $P < 0.01$). —, not determined.

parasites did not show differences in PS exposition (10) or cell growth.

LABCG2 belongs to the same subfamily as mammalian ABCG2, a well-characterized PS transporter (22) that also pumps drugs conferring a multidrug-resistant (MDR) phenotype in cancer cells (23, 24). Other *Leishmania* ABCG proteins, such as LABCG4 and LABCG6, have been described to be involved in phospholipid transport (phosphatidylcholine analogues) and drug resistance (alkyl-phospholipids) (13, 14). However, the role of the LABCG2 transporter in drug resistance has not yet been elucidated. Modulation of gene expression through gene amplification and gene deletion by homologous recombination is a common mechanism of drug resistance in *Leishmania* strains derived from both the laboratory and the field thanks to the plasticity of the *Leishmania* genome (25–27). We are therefore interested in determining whether the overexpression of LABCG2 in a *L. major* line (data not shown) could confer drug resistance. We analyzed the profile of resistance to different leishmanicidal drugs, including Sb^{III}, amphotericin B, miltefosine, pentamidine, and sitamaquine, and other compounds such as As^{III}, tafenoquine, primaquine, chloroquine, daunomycin, mefloquine, quinine, perifosine, and vinblastine (Table 1). As described previously, many of them are probably transported by other *Leishmania* ABCs (5, 13, 14, 28, 29). The results showed that promastigotes overexpressing LABCG2 were ~6- and 7-fold more resistant to As^{III} and Sb^{III}, respectively, than the control line (pUCNEO) (Table 1), suggesting that these metal ions could be substrates for the LABCG2 transporter, as previously described for MRPA and ABCI4 (3, 5). We also observed that overexpression of LABCG2 did not affect susceptibility to other metal ions such as Cd^{II}, Co^{II}, and Cu^{II} (Table 2). Contrary to other members of the *Leishmania* ABCG subfamily such as LABCG4 and LABCG6, LABCG2 does not confer resistance to the alkyl-phospholipids miltefosine and perifosine or the aminoquinolines sitamaquine and chloroquine (13, 14). Furthermore, the LABCG2 line "cured" for plasmid pUCNEO-LABCG2 (LABCG2rev) by maintaining the parasites in culture without drug selection for 3 months (LABCG2rev 90D) showed a susceptibility phenotype similar to that of the control line (Table 1).

To analyze whether the expression levels of LABCG2 were correlated with susceptibility to Sb^{III}, the LABCG2 lines were cultured in the absence of G-418 for 15, 30, 60, and 90 days to reduce plasmid copy numbers and LABCG2 expression levels (Fig. 1a). The results showed a direct relationship between LABCG2 expression levels and Sb^{III} resistance (Fig. 1b). Consequently, a greater degree of LABCG2 expression generates a higher level of resistance to Sb^{III}.

Further validation analysis showed that a second transfection event that facilitates LABCG2 overexpression in *Leishmania* (2-LABCG2) also conferred significant resistance to Sb^{III} and As^{III} (Table 2). The above-described experiments help to discard the

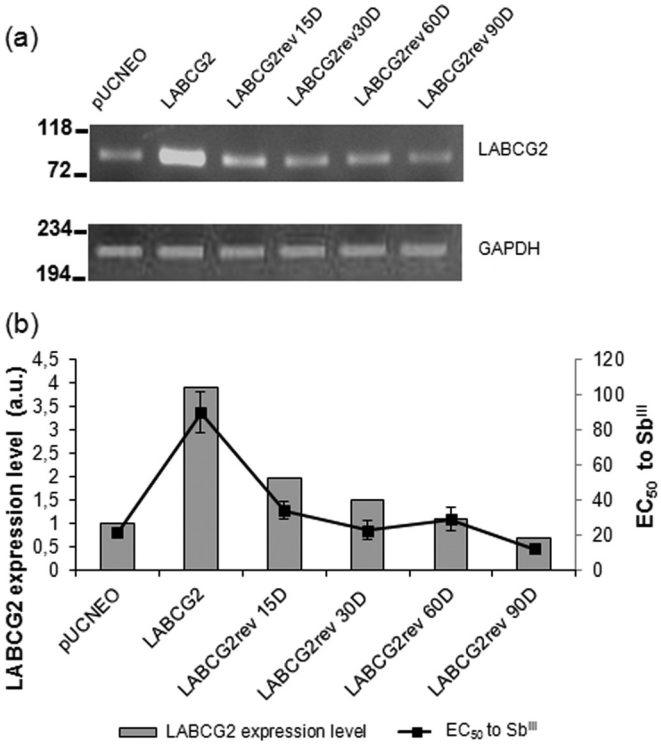

FIG 1 RNA expression analysis of LABCG2 in *L. major* lines. (a, top) LABCG2 gene expression determined by reverse transcription-PCR, as indicated by the amplified 82-bp ABCG2 fragment. (Bottom) GAPDH gene expression as the internal loading control showing the amplified 227-bp GAPDH fragment. Total RNA was extracted from the pUCNEO line (control), LABCG2 lines, and LABCG2 parasites grown for 15, 30, 60, and 90 days in the absence of G-418 pressure (LABCG2rev 15D, LABCG2rev 30D, LABCG2rev 60D, and LABCG2rev 90D lines, respectively) and then reverse transcribed to single-stranded cDNA by specific priming as described in Materials and Methods. PCR products were electrophoresed on a 4% agarose gel, stained with ethidium bromide, and viewed under a UV illuminator, and the relative intensity was measured against that of GAPDH by using a densitometer. The positions of molecular markers (base pairs) are indicated on the left. (b) Relationship between LABCG2 expression (arbitrary units [a.u.]) and Sb^{III} susceptibility (EC_{50} values ± standard deviations from three independent experiments) in *L. major* promastigote lines. Data from reverse transcription-PCR assays, representative of at least three independent experiments, are shown.

TABLE 3 Susceptibility to antimony in intracellular amastigotes of *L. major* lines[a]

Metal	Mean EC_{50} (μM) ± SD (RI)[b]		
	pUCNEO	LABCG2	LABCG2rev 90D
Sb^{III}	6.16 ± 0.07	21.30 ± 0.81 (3.4)*	5.90 ± 0.11 (0.9)
Sb^{V}	76.14 ± 2.88	>200 (>2.6)*	87.73 ± 9.71 (1.1)

[a] Macrophage-differentiated THP-1 cells infected with *L. major* lines using a macrophage/parasite ratio of 1:10 were incubated for 3 days in the presence of Sb^{III} or for 5 days in the presence of Sb^{V} at different concentrations, as described in Materials and Methods. Antimony susceptibility was determined from the percentage of infected cells and the number of intracellular amastigotes per cell in antimony-treated cultures versus nontreated cultures. Infection was determined by DAPI staining of 300 macrophages/well.

[b] Resistance indexes (RI) were calculated by dividing the EC_{50} for the *Leishmania* line overexpressing LABCG2 and LABCG2rev 90D by that for the *Leishmania* control line (pUCNEO). Data are the means ± standard deviations of results from two independent experiments. Significant differences were determined by using the Student *t* test (*, $P < 0.01$).

possibility that the resistant phenotype observed was due to an intrinsic characteristic of the clone rather than a phenotypic characteristic of the overexpression of this transporter (Table 2). The differences observed in susceptibility to Sb^{III} and As^{III} between the two transfection events involving LABCG2 were due to variations in the parasites' degree of LABCG2 expression (data not shown). Furthermore, parasites overexpressing LABCG2 fused to GFP presented a susceptibility pattern similar to that of 2-LABCG2 parasites (Table 2), demonstrating that an N-terminal fusion of the GFP tag does not interfere with the functionality of the LABCG2 transporter.

The resistance to Sb^{III} observed in the promastigote forms of *L. major* was retained in the intracellular amastigotes obtained after THP-1 cell infection (Table 3). Intracellular amastigotes overexpressing LABCG2 also presented significant resistance to sodium stibogluconate, a leishmanicidal drug containing Sb^{V}, which is reduced to Sb^{III} inside macrophages (Table 3). LABCG2 has not been described as an antimony resistance marker in previous studies based on omics techniques, similarly as described previously for *Leishmania* ABCI4 (5), considering that not every change in the expression levels of proteins involved in antimony resistance is detected by these techniques. Our findings lend weight to the idea that the overexpression of LABCG2 confers antimony resistance to *Leishmania* parasites.

Reduction in accumulation of Sb^{III} due to increased efflux in *L. major* lines overexpressing LABCG2. In order to uphold the suggestion that Sb^{III} is a potential substrate for LABCG2, the intracellular accumulation of antimony metal ions in *L. major* lines was measured after different durations (10, 20, 30, and 60 min) by ICP-MS (Fig. 2a).

Sixty minutes after incubation with Sb^{III} (Fig. 2a), parasites overexpressing LABCG2 accumulated 76% of the total amount of Sb^{III} accumulated by the control parasites. To assess whether the lower level of accumulation of this metal ion compound was due to increased efflux to the extracellular medium, *Leishmania* lines were loaded under conditions that generated similar amounts of intracellular Sb^{III}. The amount of metal ion retained inside the parasites was then measured after different periods (Fig. 2b), and so we determined that Sb^{III} efflux is faster in parasites overexpressing LABCG2 (Fig. 2b), leading to the conclusion that this transporter mediates Sb^{III} elimination. As reported previously, the primary mechanism of resistance consists of decreasing the amount of active drug within the cell by a variety of routes (30). Parasites can decrease the uptake of, increase the efflux of, or inactivate the drug by sequestration, among other possible mechanisms. The LmAQP1 aquaglyceroporin is the only protein known to transport antimony inside *L. major* (31, 32), and its downregulation subsequently leads to increased drug resistance (33). Concerning efflux, members of the eukaryotic ABCC subfamily are involved in Sb^{III} and As^{III} resistance by exporting these metal ions outside the cells or by sequestering them within intracellular vesicles (34). MRPA from *Leishmania* is one of the best-known ABC transporters implicated in antimony resistance through the sequestration of Sb^{III}-thiol complexes within an intracellular organelle near the flagellar pocket and then expulsion from the parasite by exocytosis (3). The levels of antimony efflux obtained for LABCG2 are slightly higher than the ones observed for MRPA (35) but lower than those for other MDR pumps (36, 37), suggesting similar mechanisms of action for MRPA and LABCG2. To the best of our

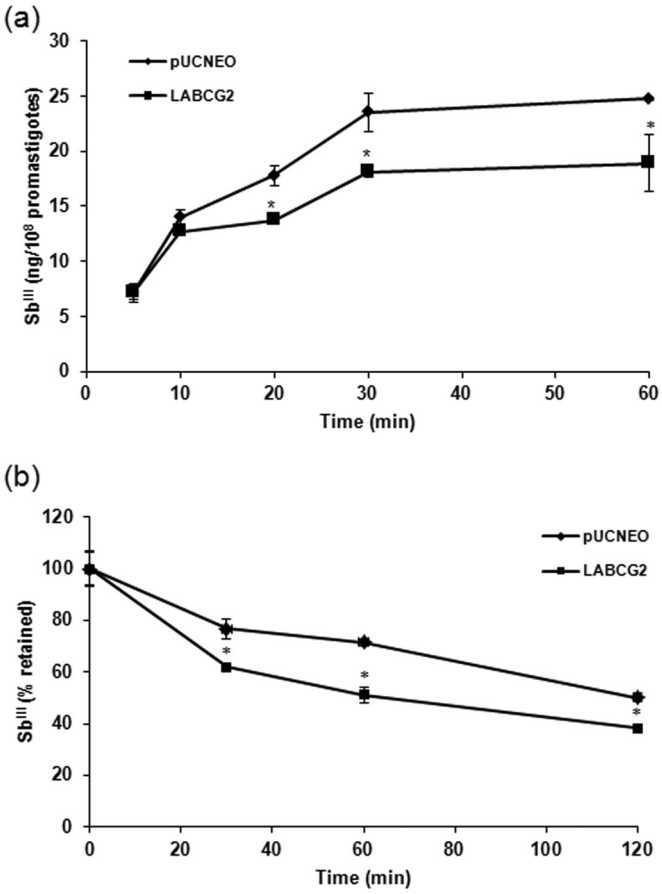

FIG 2 Time-dependent accumulation and efflux of Sb^{III} in *Leishmania* lines. (a) *L. major* lines (1×10^8 promastigotes/ml) carrying pUCNEO (control) and overexpressing LABCG2 were incubated with 100 μM Sb^{III}, and samples were taken after different time points. Antimony accumulation was measured by ICP-MS. (b) An efflux assay was performed after incubation of *Leishmania* lines with compensated concentrations of Sb^{III} for 1 h to ensure similar labeling in the different lines. The parasites were then washed and resuspended in PBS without Sb^{III} and pelleted at different time points. The data are the means ± standard deviations of results from three independent experiments performed in duplicate. Significant differences versus the control line were determined by using the Student *t* test (*, $P < 0.01$).

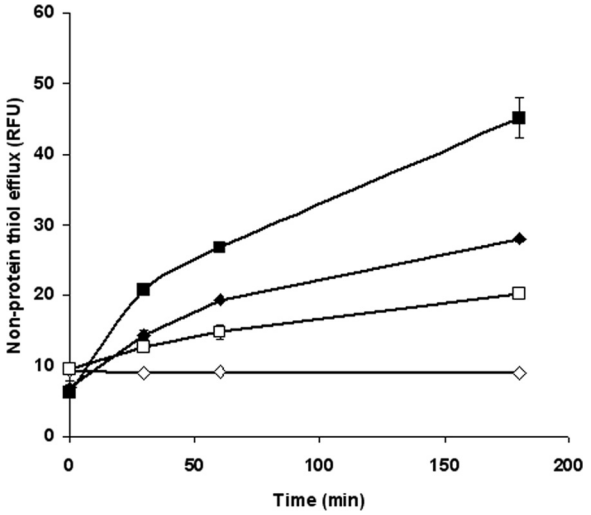

FIG 3 Nonprotein thiol efflux in *Leishmania* lines. *L. major* lines (1×10^8 promastigotes/ml) carrying pUCNEO (control) (diamonds) and overexpressing LABCG2 (squares) were incubated for 1 h either with (closed symbols) or without (open symbols) 100 μM Sb^{III} for 1 h. The promastigotes were then washed with PBS, and the supernatants were processed after different periods. Sample fluorescence (excitation, 380 nm; emission, 510 nm) was determined by using an Infinite F200 luminescence system (Tecan Austria GmbH) and expressed in relative fluorescence units (RFU). The data are the means ± standard deviations of results from three independent experiments.

knowledge, there have been no reports to suggest that the ABCG transporter is involved in Sb^{III} or As^{III} tolerance. Regarding other heavy metals, some plant full-size ABCG (PDR) transporters confer cadmium and lead tolerance (38–40). Therefore, this work presents the first description of the role of an ABCG transporter in the resistance of *Leishmania* to Sb^{III} and As^{III}.

Role of thiols in *Leishmania* ABCG2-mediated antimony resistance. As described above, thiols conjugate with heavy metals and export them to the extracellular medium, which represents an antimony resistance mechanism in *Leishmania* (41). We therefore analyzed nonprotein thiol efflux in parasites overexpressing LABCG2 using ThioStar (Fig. 3). In the presence of Sb^{III} (Fig. 3), the LABCG2 line showed significantly higher thiol efflux than did control parasites. This implies that the LABCG2 transporter confers resistance to Sb^{III} by the efflux of Sb^{III}-thiol complexes; however, we cannot discard the possibility of a cotransport activity of antimony and thiols. In the absence of Sb^{III} (Fig. 3), we also observed a significant increase in thiol efflux for parasites overexpressing the LABCG2 transporter in comparison with the control line, although the increase was not as pronounced as that in the presence of Sb^{III}. These results suggest that the LABCG2 transporter could export thiols without being conjugated to antimony.

Since conjugation of thiol adducts of Sb^{III} seems to be required for resistance to antimony, we determined the drug resistance profiles of *L. major* lines overexpressing LABCG2 after treatment with BSO, a γ-glutamylcysteine synthetase inhibitor. For these experiments, parasites were previously maintained in M199 culture medium supplemented with 10% hiFBS for 48 h at 28°C, a medium with lower levels of serum and thiols than those of RPMI 1640 medium with 20% hiFBS. We observed a significant decrease in Sb^{III} 50% effective concentrations (EC_{50}s) for parasites overexpressing LABCG2 after incubation with BSO than for those incubated without BSO (Table 4). The different EC_{50}s of Sb^{III} observed for the pUCNEO and LABCG2 lines without BSO (Table 4) with respect to values shown in Table 1 were due to differences in the contents of serum and thiols in the media used in both experiments. Hence, the Sb^{III} resistance of *L. major* lines overexpressing LABCG2 is linked to thiol levels inside the parasites, probably due to the ability of LABCG2 to export thiol-conjugated adducts, as described previously for ABCI4 (5) and MRPA (3) in *Leishmania*. The implication of ABC transporters in detoxification by the efflux of metal-thiol complexes was described previously for other organisms, such as the vacuolar transporter YCF1 in *Saccharomyces cerevisiae*, which detoxifies bis-glutathione-cadmium complexes (42), or HTM1, an ABC transporter localized in fission yeast vacuolar vesicles that confers tolerance to cadmium by taking up glutathione-derived phytochelatin conjugated to Cd^{II} (43).

TABLE 4 Susceptibility of *L. major* lines to antimony in the presence of BSO[a]

Compound	Mean EC_{50} (μM) ± SD (RI [EC_{50} decrease])	
	pUCNEO	LABCG2
Sb^{III}	6.50 ± 1.01	37.45 ± 1.50 (5.8)†
Sb^{III} + BSO	4.96 ± 1.23 (1.3)	8.23 ± 0.91 (1.7 [4.5])*

[a] Parasites were grown in M199 culture medium supplemented with 10% hiFBS for 72 h at 28°C in the presence of increasing concentrations of Sb^{III}. Cell viability was determined by using an MTT-based assay as described in Materials and Methods. Resistance indexes (RI), indicated in parentheses, were calculated by dividing the EC_{50} for the *Leishmania* line overexpressing LABCG2 by that for the *Leishmania* control line (pUCNEO) with the same treatment. The EC_{50} decrease, indicated in square brackets, was calculated by dividing the EC_{50} after Sb^{III} treatment by that for treatment with Sb^{III} plus BSO (a γ-glutamylcysteine synthetase inhibitor) in each *Leishmania* line. A total of 3 mM BSO was added to the culture medium 48 h before the susceptibility experiment was performed. The data are the means ± standard deviations of results from three independent experiments. Significant differences were determined by the Student *t* test (†, $P < 0.01$ for pUCNEO versus the LABCG2 line; *, $P < 0.01$ for the LABCG2 line treated with versus without BSO).

Considering that the LABCG2 transporter revealed an apparent capacity to export thiols in the absence of Sb^{III}, we measured internal thiol levels in *L. major* lines by flow cytometry analysis

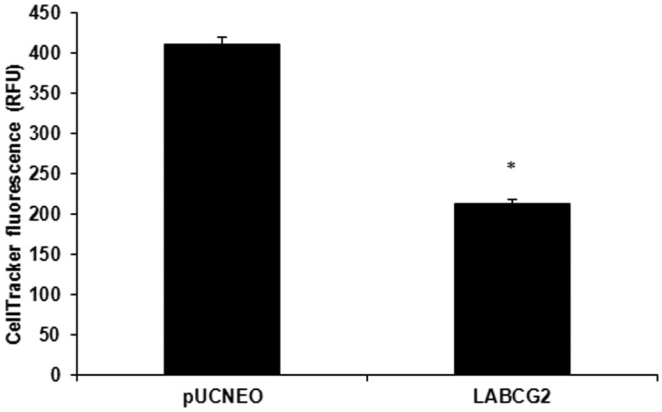

FIG 4 Determination of thiol levels in *Leishmania* lines. Promastigotes (10^7/ml) of *L. major* lines carrying pUCNEO (control) and overexpressing LABCG2 were incubated for 15 min at 37°C with 2 μM CellTracker and quantified by flow cytometry. The data are the means ± standard deviations of results from three independent experiments. Significant differences versus the control line were determined by using the Student *t* test (*, $P < 0.01$).

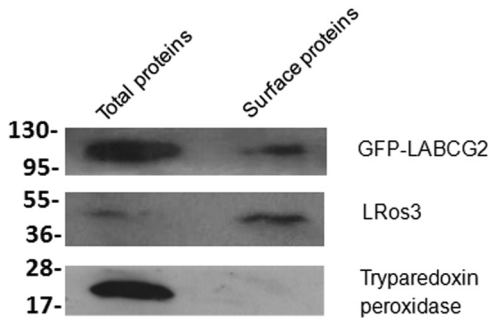

FIG 5 LABCG2 expression levels in the plasma membrane of *L. major* parasites. Biotinylated proteins from the surface of promastigotes of *Leishmania* lines expressing GFP-LABCG2 were analyzed by immunoblotting with anti-GFP, anti-LRos3 (positive control), and anti-cytosolic tryparedoxin peroxidase (negative control) antibodies. Data from Western blot assays, representative of at least three independent experiments, are shown. The positions of molecular markers (kilodaltons) are indicated on the left.

using CellTracker. LABCG2 parasites presented lower thiols level than those of the control parasites (Fig. 4). Consequently, these results support the hypothesis that LABCG2 exports thiols to the extracellular medium without the need for conjugation to antimony. Decreased levels of GSH have been observed in MDCKII cells overexpressing human ABCG2/BCRP (44). Additionally, BCRP overexpression in HN4 cells was observed to increase extracellular GSH levels (45). However, measurement of GSH transport in membrane vesicles indicated that BCRP does not catalyze any significant GSH transport (46). In contrast, ABCC1/MRP1 mediated active GSH transport in cancer cells (47). Future studies using membrane reconstitution of purified LABCG2 in proteoliposomes will further our understanding of LABCG2-mediated thiol transport in the absence of Sb^{III}.

Determination of plasma membrane localization of LABCG2 in *Leishmania* parasites using a biotinylation assay. We have previously used fluorescence microscopy assays to determine that LABCG2 partially colocalizes with the endosomal marker FM4-64 in *L. major*, suggesting that LABCG2 is located in the intracellular vesicles of the endocytic pathway of *Leishmania* parasites (10). However, we have not determined whether LABCG2 could be localized in the parasites' plasma membrane, where the transporter could be involved in the mechanism of drug efflux. We have previously described how *Leishmania* ABCI4 overexpression is localized in mitochondria, where it decreases the toxicity and accumulation of antimony, probably through efflux of the metal ion to the cytosol (5). Furthermore, ABCI4 that is localized in the parasitic plasma membrane may help to protect cells against the toxic effects of antimony and other compounds by effluxing them as conjugated thiol complexes (5).

To corroborate the possible localization of LABCG2 in the parasitic plasma membrane, we performed biotinylation assays using parasites expressing LABCG2 fused with an N-terminal GFP tag. Expression of the GFP-LABCG2 protein was determined by Western blotting of whole-parasite lysates. As expected, a band corresponding to GFP-LABCG2 was detected at ~100 kDa (Fig. 5). We also observed part of the protein localized in the PM extract, supporting the hypothesis that the transporter would be localized in intracellular vesicles, which would in turn fuse with the PM to release their content to the extracellular medium. The MRPA transporter has a similar subcellular localization, since it is found in intracellular membranes believed to correspond to vesicles that could be exocytosed via the flagellar pocket (3). Besides, we did not observe cytosolic tryparedoxin peroxidase among the biotinylated surface protein fractions, confirming that the labeling reagent did not penetrate the PM and consequently validating the specificity of the biotinylation procedure.

In conclusion, overexpression of LABCG2 in the plasma membrane of *Leishmania* may help to protect cells against the toxic effects of antimony and other compounds by effluxing them as conjugated thiol complexes. Future work should endeavor to obtain null mutants for LABCG2 that could potentially be used to understand the role of LABCG2 in *Leishmania* as a thiol-X pump and to validate it as a marker of clinical antimony resistance.

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