

# Silver and Nitrate Oppositely Modulate Antimony Susceptibility through Aquaglyceroporin 1 in *Leishmania* (*Viannia*) Species

Juvana M. Andrade,<sup>a</sup> Elio H. Baba,<sup>a</sup> Ricardo A. Machado-de-Avila,<sup>b,c</sup> Carlos Chavez-Olortegui,<sup>c</sup> Cynthia P. Demicheli,<sup>d</sup> Frédéric Frézard,<sup>c</sup> Rubens L. Monte-Neto,<sup>a</sup> Silvane M. F. Murta<sup>a</sup>

Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG, Brazil<sup>a</sup>; Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil<sup>b</sup>; Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil<sup>c</sup>; Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil<sup>d</sup>

**Antimony (Sb) resistance in leishmaniasis chemotherapy has become one of the major challenges to the control of this spreading worldwide public health problem. Since the plasma membrane pore-forming protein aquaglyceroporin 1 (AQP1) is the major route of Sb uptake in *Leishmania*, functional studies are relevant to characterize drug transport pathways in the parasite. We generated AQP1-overexpressing *Leishmania guyanensis* and *L. braziliensis* mutants and investigated their susceptibility to the trivalent form of Sb (Sb<sup>III</sup>) in the presence of silver and nitrate salts. Both AQP1-overexpressing lines presented 3- to 4-fold increased AQP1 expression levels compared with those of their untransfected counterparts, leading to an increased Sb<sup>III</sup> susceptibility of about 2-fold. Competition assays using silver nitrate, silver sulfadiazine, or silver acetate prior to Sb<sup>III</sup> exposure increased parasite growth, especially in AQP1-overexpressing mutants. Surprisingly, Sb<sup>III</sup>-sodium nitrate or Sb<sup>III</sup>-potassium nitrate combinations showed significantly enhanced antileishmanial activities compared to those of Sb<sup>III</sup> alone, especially against AQP1-overexpressing mutants, suggesting a putative nitrate-dependent modulation of AQP1 activity. The intracellular level of antimony quantified by graphite furnace atomic absorption spectrometry showed that the concomitant exposure to Sb<sup>III</sup> and nitrate favors antimony accumulation in the parasite, increasing the toxicity of the drug and culminating with parasite death. This is the first report showing evidence of AQP1-mediated Sb<sup>III</sup> susceptibility modulation by silver in *Leishmania* and suggests the potential antileishmanial activity of the combination of nitrate salts and Sb<sup>III</sup>.**

Leishmaniasis is one of six diseases regarded by the World Health Organization to be major threats to developing countries. This complex disease is caused by different species of protozoan parasites belonging to the genus *Leishmania*. This disease is endemic in 98 countries, with approximately 350 million people being at risk and an estimated 12 million people being infected worldwide. Its clinical manifestations range from self-healing skin lesions in cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) to visceral leishmaniasis (VL), which is lethal if left untreated. In the New World, *Leishmania* (*Viannia*) *guyanensis* and *L. (Viannia) braziliensis* cause both CL and MCL (1, 2). Approximately 0.7 million to 1.2 million cases of CL and 0.2 million to 0.4 million cases of VL are diagnosed each year (3). In the absence of an effective vaccine for humans, chemotherapy is still the main strategy used to control the disease; however, the emergence of drug-resistant *Leishmania* parasites has become a challenge for antimony-based leishmaniasis chemotherapy. The epicenter of antimony-resistant visceral leishmaniasis has been found to be in India, but antimony-unresponsive cases are reported worldwide, and these include cutaneous cases in the New World (4). Miltefosine (hexadecylphosphocholine) and amphotericin B (AMB), a polyene macrolide, have been chosen as first-line alternative drugs to treat leishmaniasis in the Indian subcontinent, where VL is endemic (5, 6). Although the evidence is scarce, recent evidence of clinical miltefosine unresponsiveness (7), cases of relapse after AMB treatment (8), and the selection of AMB-resistant *Leishmania* field isolates (9) indicate the current major threats of long-term drug exposure.

Pentavalent antimony-containing compounds (Sb<sup>V</sup>) such as sodium stibogluconate (Pentostam) and *N*-methyl-glucamine (Glucantime) made from sodium or potassium hexahydroxoan-

timonate salts have been used as first-line therapies against all forms of leishmaniasis. They are prodrugs and must be reduced *in vivo* to the trivalent active form (Sb<sup>III</sup>) (10).

The major entry route of Sb<sup>III</sup> in *Leishmania* parasites is through aquaglyceroporin 1 (AQP1), a six-helix plasma transmembrane pore-forming protein that also allows the transport of water, glycerol, urea, and other small uncharged solutes and that thus plays an important role in osmoregulation (11). At physiological pH, the affinity of trivalent antimony through AQP1 is explained by its uncharged Sb(OH)<sub>3</sub> form, which is structurally similar to glycerol (12). AQP1-mediated antimony sensitivity is species specific, with cutaneous leishmaniasis-associated species being more sensitive to the drug. This phenotype is a result of posttranscriptionally regulated AQP1 expression (13). Although the molecular basis of the mechanisms of resistance are not fully understood, the downregulation, mutation, and deletion of the AQP1-encoding gene have been clearly associated with Sb resis-

Received 7 April 2016 Returned for modification 19 April 2016

Accepted 30 April 2016

Accepted manuscript posted online 9 May 2016

Citation Andrade JM, Baba EH, Machado-de-Avila RA, Chavez-Olortegui C, Demicheli CP, Frézard F, Monte-Neto RL, Murta SMF. 2016. Silver and nitrate oppositely modulate antimony susceptibility through aquaglyceroporin 1 in *Leishmania* (*Viannia*) species. *Antimicrob Agents Chemother* 60:4482–4489. doi:10.1128/AAC.00768-16.

Address correspondence to Rubens L. Monte-Neto, rubens.neto@cpqrr.fiocruz.br, or Silvane M. F. Murta, silvane@cpqrr.fiocruz.br.

R.L.M.-N. and S.M.F.M. contributed equally to this article.

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tance in both laboratory-selected and field isolates of *Leishmania* (14–16). In *Leishmania*, AQP1 has also been implicated in the transport of the Sb-related metal arsenite ( $\text{As}^{\text{III}}$ ) (17). Indeed, disruption of the AQP1 homolog in the *Leishmania*-related parasite *Trypanosoma brucei* (the etiological agent of human African trypanosomiasis), *TbAQP2*, generates melarsoprol (As-based drug)-resistant parasites, and laboratory-selected melarsoprol-resistant trypanosomes also showed a disrupted *TbAQP2* (18). In fact, *T. brucei gambiense* field isolates with reduced susceptibility to melarsoprol harbor *TbAQP2* mutations and gene loss (19). A similar functional correlation was demonstrated in *L. mexicana*, in which the heterologous expression of *TbAQP2* increased the sensitivity of arsenicals >1,000-fold (20).

We have recently proposed theoretical transport models for the characterization of Sb resistance by considering different pathways where decreased drug influx plays a critical role (21). Despite biophysical characterization, pharmacological approaches are useful for the study of drug resistance mechanisms in *Leishmania*. In this regard, specific AQP1 modulators would support functional *in vitro* drug transport and competition studies. The silver-containing compounds silver nitrate ( $\text{AgNO}_3$ ) and silver sulfadiazine (SAG) are known to inhibit plant aquaporins and human aquaporin 1 (hAQP1) more effectively than gold- or mercury-containing compounds (22). Here, we performed a functional analysis of the AQP1 transporter gene in *Leishmania* (*Viannia*) species and investigated the role of silver and nitrate on  $\text{Sb}^{\text{III}}$  susceptibility in wild-type (WT) and AQP1-overexpressing parasites.

## MATERIALS AND METHODS

**Parasite cultures.** Promastigote forms of *L. (Viannia) guyanensis* (IUMB/BR/85/M9945) and *L. (Viannia) braziliensis* (MHOM/BR/75/M2904) were cultured *in vitro* at 26°C in medium M199 supplemented as described by Liarte and Murta (23).

**Transfection of *L. guyanensis* and *L. braziliensis* with *LbAQP1*.** pIR1-BSD-LbAQP1 was constructed as described previously using blastidicin S deaminase (BSD) as a selectable marker (24). An 858-bp segment corresponding to the open reading frame of *L. braziliensis AQP1* (*LbAQP1*; *L. braziliensis* M.31.0020; genome version 2013-01-16, available at tritrypdb.org) was amplified using forward primer 5'-tAGATCTccaccATGGCGATTGAAAACCACAT-3' and reverse primer 5'-ttAGATCTCTACGCACCGCTCGGTATTA-3', in which the underlined sequences correspond to the BgIII restriction site and the lowercase nucleotides correspond to the Kozak sequence. The PCR product obtained was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced for confirmation of the correct fragment, and subcloned into the pIR1-BSD expression vector. The constructs pIR1-BSD (empty vector) and pIR1-BSD-LbAQP1 were linearized by *Swa*I digestion and electroporated into *L. braziliensis* and *L. guyanensis* lines using a GenePulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA). This stable transfection allowed integration of the vector into the 18S ribosomal DNA small-subunit locus of the parasite (25).

**Production of polyclonal antiserum and Western blotting.** Polyclonal antibodies against *LbAQP1* were obtained in order to monitor protein overexpression. Representative peptides of aquaglyceroporin 1 were analyzed by the use of two predictors for B cells: the BEPIPRED and BCPRED12 programs (26). In addition, the sequences were subjected to analysis using SIGCLEAVE software for the identification of the signal peptide and TMHMM software to check the presence of transmembrane domains. The peptides QHFDDAGVMLLPNETMASKFSGVFVT and NPTRDLGPRIFTAMLWGKEPFTLHG are considered highly immunogenic. Both peptides were synthesized using the 9-fluorenylmethoxy carbonyl synthesis technique developed by Merrifield (27). Polyclonal antiserum was produced in New Zealand White rabbits, whose use was

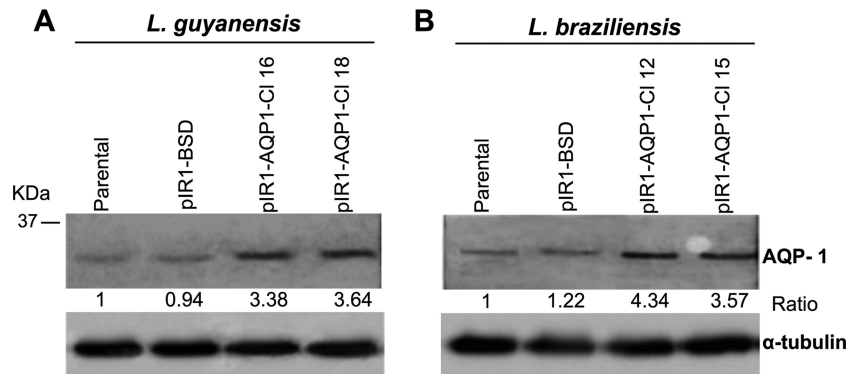
approved by the appropriate ethical committee for animal research (CEUA-375/2012, Universidade Federal de Minas Gerais). The rabbits were inoculated with Freund's adjuvant (Sigma, St. Louis, MO, USA) containing 1,500  $\mu\text{g}$  of both synthetic peptides on days 0, 15, and 30. The rabbits received three subcutaneous inoculations, the first with complete Freund's adjuvant and the other two with incomplete Freund's adjuvant. Serum was obtained 10 and 20 days after the last inoculation.

Western blotting was carried out to investigate the AQP1 protein levels in transfected *Leishmania* clonal lines. Protein extracts from *L. braziliensis* and *L. guyanensis* clonal lines were obtained as previously described (28). The protein concentration was determined by the Bradford method. Proteins (100  $\mu\text{g}$ ) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. The blots were incubated overnight in the presence of the anti-AQP1 antiserum at a dilution of 1:5. Membranes were then incubated for 1 h at 25°C with alkaline phosphatase-conjugated affinity-purified anti-rabbit IgG (Sigma, St. Louis, MO, USA) at a 1:6,000 dilution. The bands on the nitrocellulose membrane were then visualized by staining with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

**$\text{Sb}^{\text{III}}$  and  $\text{AgNO}_3$  susceptibility assays.** The effective concentration required to decrease growth by 50% ( $\text{EC}_{50}$ ) for  $\text{Sb}^{\text{III}}$  was determined using a Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA), as described by Andrade and Murta (24). Promastigotes of wild-type *L. guyanensis* and *L. braziliensis* clonal lines transfected with the construct pIR1-BSD (empty vector) or pIR1-BSD-LbAQP1 or untransfected clonal lines were submitted to the  $\text{Sb}^{\text{III}}$  susceptibility test. Parasites were seeded in medium M199 at  $2 \times 10^6$  cells  $\text{ml}^{-1}$  in 24-well plates in the presence of several concentrations of  $\text{Sb}^{\text{III}}$  (potassium antimonyl tartrate with Sb at concentrations of up to 3 mM) and  $\text{AgNO}_3$  (0.125 to 10  $\mu\text{M}$ ) for 48 h.  $\text{EC}_{50}$ s were determined from three independent measurements performed in triplicate, using the linear interpolation method (29).

**Competition assays.** Since  $\text{AgNO}_3$  has been described to be a potential inhibitor of AQP1 (22), we performed competition assays using different sources of silver or nitrate salts ( $\text{AgNO}_3$ , SAG,  $\text{AgC}_2\text{H}_3\text{O}_2$ ,  $\text{NaNO}_3$ ,  $\text{KNO}_3$ ) concomitantly or not with  $\text{Sb}^{\text{III}}$  (potassium antimonyl tartrate) to better investigate the role of silver and/or nitrate on the modulation of AQP1 activity. All reagents were purchased from Sigma and were of the purest grade available. First, we determined the  $\text{EC}_{50}$  of each compound. For competition assays,  $2 \times 10^6$  parasites were seeded into 24-well cell culture plates containing medium M199. Subsequently, the  $\text{EC}_{50}$  of each compound ( $\text{AgNO}_3$ , SAG,  $\text{AgC}_2\text{H}_3\text{O}_2$ ,  $\text{NaNO}_3$ , or  $\text{KNO}_3$ ) was added to the parasite culture 5 min prior to  $\text{Sb}^{\text{III}}$  treatment (with the corresponding  $\text{Sb}^{\text{III}}$   $\text{EC}_{50}$ ; see Table 1), followed by incubation for 48 h. Growth as a percentage of that of the parental WT was determined by automated cell counting using a Z1 Coulter Counter.

**Antimony uptake.** The intracellular accumulation of antimony was measured as previously reported (30). Briefly, log-phase *Leishmania* promastigotes were washed twice with HEPES-glucose (HG) buffer (20 mM HEPES, 0.15 M NaCl, 10 mM glucose, pH 7.2) and resuspended in this buffer at a density of  $1.0 \times 10^8$  cells/ml. This parasite suspension was aliquoted (1 ml) and placed in quadruplicate into tubes containing (i) parasites only (blank), (ii) parasites and 540  $\mu\text{M}$   $\text{Sb}^{\text{III}}$  (potassium antimonyl tartrate, positive control), (iii) parasites and 540  $\mu\text{M}$   $\text{Sb}^{\text{III}}$  plus 30  $\mu\text{M}$   $\text{AgC}_2\text{H}_3\text{O}_2$ , (iv) parasites and 540  $\mu\text{M}$   $\text{Sb}^{\text{III}}$  plus 160  $\mu\text{M}$   $\text{NaNO}_3$ , and (v) parasites and 540  $\mu\text{M}$   $\text{Sb}^{\text{III}}$  plus 120  $\mu\text{M}$   $\text{KNO}_3$ . Parasites treated only with  $\text{Sb}^{\text{III}}$  and silver or nitrate salt were used as controls. Cells were then incubated for 1 h, centrifuged, and washed three times with HG buffer. The pellets were then resuspended in 100  $\mu\text{l}$  of HG buffer. One aliquot of 10  $\mu\text{l}$  from each tube was used for normalization (parasite quantification); after the last centrifugation, the pellet was resuspended in 100  $\mu\text{l}$  of nitric acid (65%) for cell digestion. The intracellular antimony content was quantified by graphite furnace atomic absorption spectrometry (AAnalyst 600; PerkinElmer). Each uptake assay was performed three times, and the Sb



**FIG 1** *LbAQP1* expression levels in *Leishmania* (*Viannia*) species. (A) *L. guyanensis* and (B) *L. braziliensis*. Proteins (100  $\mu$ g) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. The blots were probed with an anti-AQP1 antiserum at a dilution of 1:5 and with alkaline phosphatase-conjugated affinity-purified anti-rabbit IgG at a 1:6,000 dilution and developed with NBT-BCIP. First lane in each panel, untransfected parental wild-type strains; second lane in each panel, parental wild type transfected with pIR1-BSD (empty expression vector); third and fourth lanes in each panel, clonal lines (CI) constitutively expressing *LbAQP1* (transfected with the expression vector pIR1-BSD containing *LbAQP1* 18S ribosomal small-subunit DNA integration cassette). Densitometric analysis of the bands on the Western blot was performed by normalization to the  $\alpha$ -tubulin antibody signal and performed using ImageJ software (version 1.48).

dosage from blanks was used for background subtraction. The total Sb content was normalized by  $1.0 \times 10^8$  promastigote forms.

## RESULTS

**Overexpression of AQP1 in *Leishmania* spp.** In order to evaluate whether the overexpression of AQP1 interferes with the Sb<sup>III</sup> resistance phenotype, *L. guyanensis* and *L. braziliensis* lines were transfected with the *LbAQP1* gene. To confirm the transfection, genomic DNA from the transfected clones was subjected to PCR using primers specific for the *BSD* gene, which confers resistance to blasticidin (data not shown) (31). *Leishmania* clones PCR positive for the *BSD* gene were subjected to a Western blot assay to assess the AQP1 expression levels in the transfected parasites. The AQP1 expression level in *LbAQP1*-overexpressing *L. braziliensis* and *L. guyanensis* (wild-type background) was 3.3- to 4.3-fold higher than that in the nontransfected parental counterpart. AQP1 expression levels were normalized by the level of expression of  $\alpha$ -tubulin (Fig. 1). These transfected clones were subjected to an Sb<sup>III</sup> susceptibility test and competition assays using silver and nitrate salts.

**Susceptibility of *Leishmania* spp. to potassium antimonyl tartrate (Sb<sup>III</sup>) and silver nitrate.** Antileishmanial drug susceptibility assays were carried out in order to evaluate the Sb<sup>III</sup> and

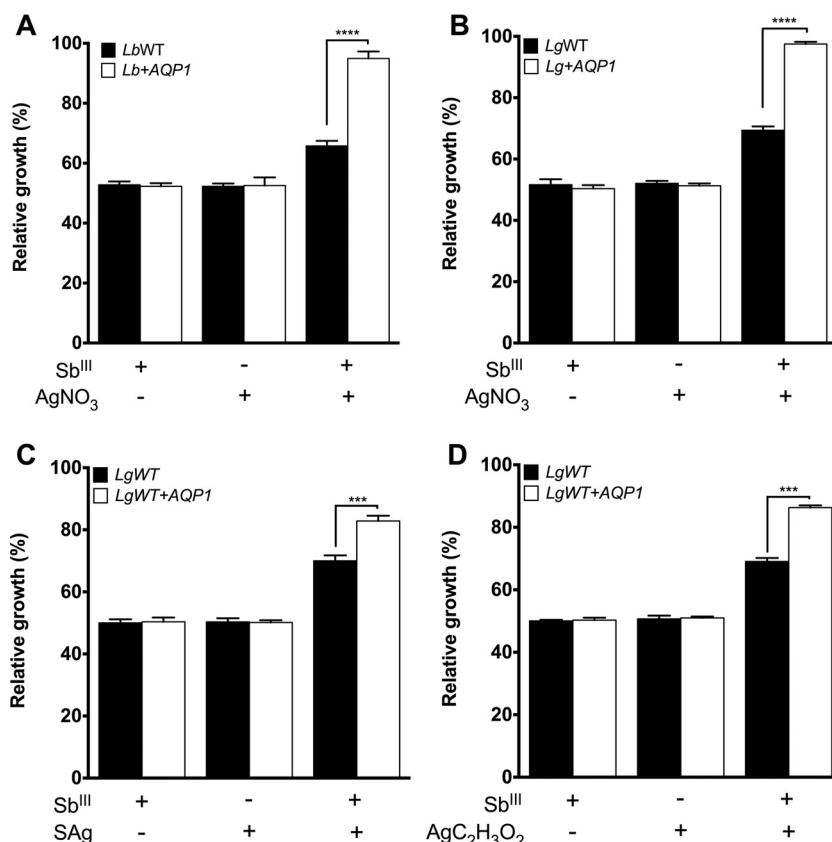
AgNO<sub>3</sub> sensitivity phenotypes of *L. guyanensis* and *L. braziliensis*. Table 1 depicts the half-maximal effective concentration (EC<sub>50</sub>) values for the different *Leishmania* lines overexpressing or not expressing AQP1. Although they are members of the same subgenus, *L. guyanensis* was two times more sensitive to Sb<sup>III</sup> than *L. braziliensis*, presenting EC<sub>50</sub>s of 48.72 and 108  $\mu$ M, respectively. The overexpression of AQP1 in both *L. guyanensis* and *L. braziliensis* resulted in 1.6-fold and 2.3- to 2.6-fold greater susceptibility to Sb<sup>III</sup>, respectively, with the corresponding susceptibility indexes (SIs) being approximately 0.6 and 0.4 (Table 1). Upon AgNO<sub>3</sub> treatment, AQP1-overexpressing mutants presented a slightly increased resistance phenotype, with the *L. guyanensis* and *L. braziliensis* mutants showing SIs of approximately 1.2 and 1.6, respectively (Table 1). This observation suggests that AQP1 would also behave as a target for AgNO<sub>3</sub> in *Leishmania*. Additionally, AgNO<sub>3</sub> was highly toxic against *Leishmania* parasites, showing EC<sub>50</sub>s of approximately 1  $\mu$ M (Table 1). The drug susceptibilities of parasites transfected with the empty vector were not different from those of their wild-type counterparts.

**Combined Sb<sup>III</sup>-silver or Sb<sup>III</sup>-nitrate decreases or enhances antimony sensitivity in *Leishmania*, respectively.** Since AgNO<sub>3</sub> has been described to be an aquaporin inhibitor, we performed a

**TABLE 1** EC<sub>50</sub>s of Sb<sup>III</sup> and AgNO<sub>3</sub> and corresponding SIs for wild-type or *LbAQP1*-overexpressing *Leishmania* (*Viannia*) species<sup>a</sup>

<i>Leishmania</i> line	Sb <sup>III</sup>		AgNO <sub>3</sub>	
	EC <sub>50</sub> (95% CI) ( $\mu$ M)	SI	EC <sub>50</sub> (95% CI) ( $\mu$ M)	SI
<i>L. guyanensis</i> WT	48.72 (43.09–55.08)		1.37 (1.25–1.49)	
<i>L. guyanensis</i> /pIR1-BSD	48.96 (42.79–56.02)		1.4 (1.28–1.51)	
<i>L. guyanensis</i> /pIR1-BSD-AQP1 cl 16	30.09 (27.21–33.26)**	0.61	1.62 (1.47–1.8)*	1.18
<i>L. guyanensis</i> /pIR1-BSD-AQP1 cl 18	29.50 (26.12–33.31)**	0.6	1.61 (1.48–1.74)*	1.17
<i>L. braziliensis</i> WT	108 (103.3–112.9)		0.95 (0.88–1.03)	
<i>L. braziliensis</i> /pIR1-BSD	94.21 (88.06–100.8)		0.94 (0.86–1.02)	
<i>L. braziliensis</i> /pIR1-BSD-AQP1 cl 12	40.94 (37.94–44.17)***	0.38	1.5 (1.4–1.62)***	1.56
<i>L. braziliensis</i> /pIR1-BSD-AQP1 cl 15	46 (43.05–49.13)***	0.42	1.57 (1.47–1.67)***	1.65

<sup>a</sup> Abbreviations and symbols: CI, confidence interval; cl, clone; SI, susceptibility index, which is equal to the EC<sub>50</sub> for the *LgAQP1*-overexpressing isolate/EC<sub>50</sub> for the WT isolate; \*\*\*,  $P < 0.0001$ ; \*\*,  $P = 0.0012$ ; \*,  $P = 0.0121$  (analysis of variance). EC<sub>50</sub>s are averages from at least two independent experiments performed in triplicate and were obtained after 48 h of exposure to potassium antimonyl tartrate, which was used as the Sb<sup>III</sup> source (Sigma-Aldrich, St. Louis, MO, USA), or AgNO<sub>3</sub> (silver nitrate).

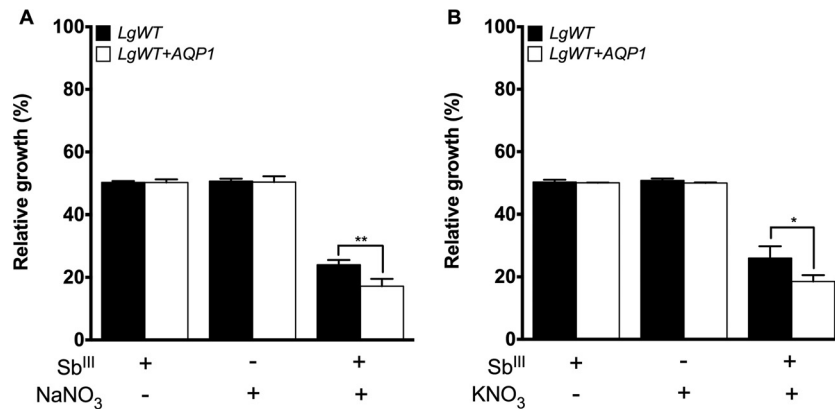


**FIG 2** Effect of silver nitrate ( $\text{AgNO}_3$ ) (A and B), silver sulfadiazine (SAG) (C), and silver acetate ( $\text{AgC}_2\text{H}_3\text{O}_2$ ) (D) on the growth of *Leishmania* (*Viannia*) species upon  $\text{Sb}^{\text{III}}$  exposure. (A) *L. braziliensis*; (B to D) *L. guyanensis*. Parasites ( $2 \times 10^6$ ) were seeded into 24-well cell culture plates containing medium M199. Cells were exposed to the  $\text{EC}_{50}$ s of  $\text{Sb}^{\text{III}}$  and  $\text{AgNO}_3$  (Table 1), SAG (3.0 and 3.5  $\mu\text{M}$  for WT *L. guyanensis* and WT *L. guyanensis* overexpressing AQP1, respectively), and  $\text{AgC}_2\text{H}_3\text{O}_2$  (3.1 and 3.6  $\mu\text{M}$  for WT *L. guyanensis* and WT *L. guyanensis* overexpressing AQP1, respectively) independently or were treated with the silver salt 5 min prior to  $\text{Sb}^{\text{III}}$  exposure, followed by incubation for 48 h. Parasite numbers were quantified by cell counting using a Coulter Counter. The data represent the means  $\pm$  standard errors from three independent experiments performed in triplicate. Statistical analysis was carried out using Student's *t* test and one-way analysis of variance followed by Bonferroni's multiple-comparison test. \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ . LbWT, *L. braziliensis* WT; Lb+AQP1, *L. braziliensis*/pIR1-BSD-LbAQP1 clone 12; LgWT, *L. guyanensis* WT; Lg+AQP1, *L. guyanensis*/pIR1-BSD-LbAQP1 clone 18. The growth of *Leishmania* mutants transfected with pIR1-BSD only (empty vector) was similar to that of the parental WT (data not shown).

competition assay using  $\text{AgNO}_3$  concomitantly with or not concomitantly with  $\text{Sb}^{\text{III}}$  treatment. Similar growth profiles were observed when the *L. guyanensis* and *L. braziliensis* WT strains were exposed to the drug combinations, leading to reduced sensitivity (Fig. 2A and B). This behavior could be explained by a reduced  $\text{Sb}^{\text{III}}$  activity influenced by  $\text{AgNO}_3$ . In order to investigate the role of AQP1, AQP1-overexpressing *L. guyanensis* and *L. braziliensis* mutants were submitted to a competition assay, as described above. Both *L. guyanensis* and *L. braziliensis* AQP1-overexpressing mutants became resistant at 48 h under  $\text{AgNO}_3$ - $\text{Sb}^{\text{III}}$  exposure. Indeed, all LbAQP1-overexpressing mutants presented results statistically significantly different from those for the WT strains when they were exposed to the drug combination (Fig. 2).

These results show evidence of drug susceptibility modulation by the  $\text{AgNO}_3$  and  $\text{Sb}^{\text{III}}$  combination mediated by AQP1. To investigate the role of silver on the modulation of susceptibility mediated by AQP1, we performed functional competition assays with WT and AQP1-overexpressing *L. guyanensis* lines using different silver and nitrate sources. The silver sulfadiazine (SAG)  $\text{EC}_{50}$ s were 3 and 3.5  $\mu\text{M}$  for the wild-type and AQP1-overexpressing *L. guyanensis* lines, respectively. These values were very

similar to the silver acetate  $\text{EC}_{50}$ s for both lines, which were 3.1  $\mu\text{M}$  and 3.6  $\mu\text{M}$ , respectively. On the other hand, nitrate salts were less toxic to the *Leishmania* lines than the silver salts. The potassium nitrate  $\text{EC}_{50}$ s were 120 and 160  $\mu\text{M}$  for the wild-type and AQP1-overexpressing *L. guyanensis* lines, respectively, whereas the sodium nitrate  $\text{EC}_{50}$  was 160  $\mu\text{M}$  for both *Leishmania* lines. When both parasites were exposed to the  $\text{Sb}^{\text{III}}$ -silver sulfadiazine or  $\text{Sb}^{\text{III}}$ -silver acetate drug combination, they became more resistant to  $\text{Sb}^{\text{III}}$  (Fig. 2C and D), as was observed for  $\text{Sb}^{\text{III}}$ -silver nitrate. Moreover, this increased  $\text{Sb}^{\text{III}}$  resistance was higher in AQP1-overexpressing *L. guyanensis* parasites than in WT *L. guyanensis* (Fig. 2C and D, white bars). Surprisingly, a pronounced antileishmanial activity was observed when both WT and AQP1-overexpressing *L. guyanensis* parasites were exposed to the  $\text{Sb}^{\text{III}}$ -sodium nitrate and  $\text{Sb}^{\text{III}}$ -potassium nitrate combinations (Fig. 3A and B). Additionally, this increased  $\text{Sb}^{\text{III}}$  susceptibility was higher in AQP1-overexpressing *L. guyanensis* parasites than in the WT *L. guyanensis* parasites (Fig. 3C and D), suggesting the involvement of AQP1 in this activity. No change in the relative growth of parasites incubated only with the  $\text{EC}_{50}$  of each nitrate salt was observed (Fig. 3).



**FIG 3** Effect of sodium nitrate (NaNO<sub>3</sub>) and potassium nitrate (KNO<sub>3</sub>) on the growth of *L. (Viannia) guyanensis* lines upon Sb<sup>III</sup> exposure. Parasites ( $2 \times 10^6$ ) were seeded into 24-well cell culture plates containing medium M199. Cells were exposed to the EC<sub>50</sub> of Sb<sup>III</sup> (48.72 and 29.50  $\mu$ M for the *L. guyanensis* WT and the *L. guyanensis* WT overexpressing AQP1, respectively) and the EC<sub>50</sub> of each compound (160  $\mu$ M NaNO<sub>3</sub> for both lines; 120 and 160  $\mu$ M KNO<sub>3</sub> for the *L. guyanensis* WT and the *L. guyanensis* WT overexpressing AQP1, respectively) independently or were treated with each compound for 5 min prior to Sb<sup>III</sup> exposure, followed by incubation for 48 h. Parasite numbers were quantified by cell counting using a Coulter Counter. The data represent the means  $\pm$  standard errors from three independent experiments performed in triplicate. Statistical analysis was carried out using Student's *t* test and one-way analysis of variance followed by Bonferroni's multiple-comparison test. \*,  $P < 0.04$ ; \*\*,  $P < 0.001$ . LgWT, *L. guyanensis* WT; Lg+AQP1, *L. guyanensis*/pIR1-BSD-LbAQP1 clone 18.

In order to further investigate this antileishmanial activity, an Sb<sup>III</sup>-resistant *L. guyanensis* line was used in this competition assay. This resistant line was previously selected *in vitro* by stepwise increased Sb<sup>III</sup> pressure and is 19-fold more resistant than its WT susceptible counterpart (23). One of the promising findings here is that Sb<sup>III</sup>-resistant parasites were also susceptible to treatment with the Sb<sup>III</sup>-sodium nitrate and Sb<sup>III</sup>-potassium nitrate combinations, similar to the findings for WT *L. guyanensis* (Fig. 4A and B). This result indicates that the combination of nitrate salts and Sb<sup>III</sup> is toxic for *Leishmania*, even Sb<sup>III</sup>-resistant parasites, reverting this phenotype and leading to antimony resensitization.

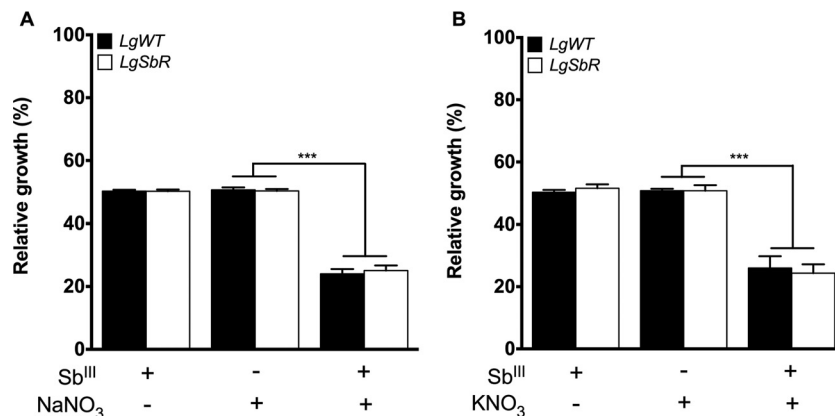
**The Sb<sup>III</sup>-nitrate combination favors intracellular antimony accumulation in *Leishmania guyanensis*.** We evaluated the level of antimony uptake in wild-type *L. guyanensis* parasites incubated in the presence of Sb<sup>III</sup> and silver or nitrate salts. Interestingly, parasites treated with Sb<sup>III</sup>-sodium nitrate and Sb<sup>III</sup>-potassium

nitrate exhibited higher intracellular antimony concentrations than parasites exposed only to Sb<sup>III</sup> (control) and Sb<sup>III</sup>-silver acetate (Fig. 5). This result shows that the combination of Sb<sup>III</sup> and nitrate favors Sb<sup>III</sup> accumulation in the parasite, increasing drug toxicity and leading to parasite death. Further investigations are needed to mechanistically explore the transport pathways involved in this accumulation of increased amounts of antimony.

In order to investigate the formation of novel chemical entities in culture medium upon exposure to antimony tartrate and nitrate salts combined, mass spectrometry analysis was conducted; however, no complexes were identified (data not shown).

## DISCUSSION

Drug resistance is one of the major barriers to successful leishmaniasis chemotherapy. Molecular mechanisms of antimony resistance in *Leishmania* are complex and multifactorial and take



**FIG 4** Effect of sodium nitrate (NaNO<sub>3</sub>) and potassium nitrate (KNO<sub>3</sub>) on the growth of *L. (Viannia) guyanensis* wild-type (LgWT) and Sb<sup>III</sup>-resistant (LgSbR) lines upon Sb<sup>III</sup> exposure. Parasites ( $2 \times 10^6$ ) were seeded into 24-well cell culture plates containing medium M199. Cells were exposed to the EC<sub>50</sub> of Sb<sup>III</sup> (48.72 and 912  $\mu$ M for the *L. guyanensis* WT and the Sb<sup>III</sup>-resistant *L. guyanensis*, respectively) and the EC<sub>50</sub> of each compound (160 and 200  $\mu$ M NaNO<sub>3</sub> for the *L. guyanensis* WT and Sb<sup>III</sup>-resistant *L. guyanensis*, respectively; 120 and 180  $\mu$ M KNO<sub>3</sub> for the *L. guyanensis* WT and Sb<sup>III</sup>-resistant *L. guyanensis*, respectively) independently or were treated with each compound for 5 min prior to Sb<sup>III</sup> exposure, followed by incubation for 48 h. Parasite numbers were quantified by cell counting using a Coulter Counter. The data represent the means  $\pm$  standard errors from three independent experiments performed in triplicate. Statistical analysis was carried out using Student's *t* test and one-way analysis of variance followed by Bonferroni's multiple-comparison test. \*\*\*,  $P < 0.0001$ .

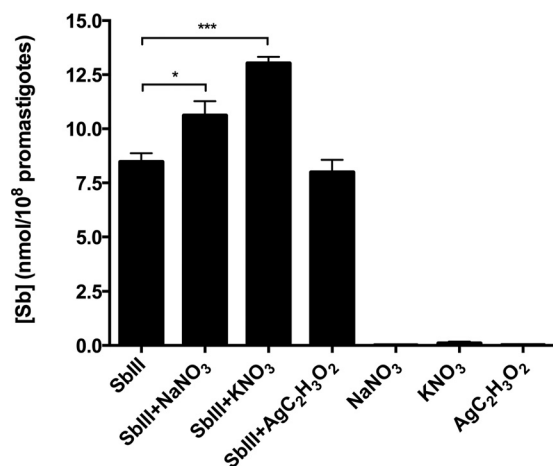


FIG 5 Levels of antimony in promastigote forms of *L. (Viannia) guyanensis* wild-type (LgWT) incubated in the presence of Sb<sup>III</sup>, Sb<sup>III</sup>-sodium nitrate (NaNO<sub>3</sub>), Sb<sup>III</sup>-potassium nitrate (KNO<sub>3</sub>), or Sb<sup>III</sup>-silver acetate (AgC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>). As a control, the parasites were incubated only with sodium nitrate, potassium nitrate, or silver acetate. The intracellular level of antimony was quantified by graphite furnace atomic absorption spectrometry. The data represent the means  $\pm$  standard errors from three independent experiments performed in quadruplicate. Statistical analysis was carried out using Student's *t* test and one-way analysis of variance followed by Bonferroni's multiple-comparison test. \*,  $P < 0.001$ ; \*\*\*,  $P < 0.0004$ .

place by several pathways, such as entry, metabolism, efflux, sequestration, and cell death (32). Since the plasma membrane pore-forming protein aquaglyceroporin 1 (AQP1) is the major route of Sb uptake in *Leishmania*, we performed functional studies to characterize drug transport pathways in the parasite. In order to investigate AQP1 involvement in this process, AQP1-overexpressing *L. guyanensis* and *L. braziliensis* mutants were obtained and treated with Sb<sup>III</sup> in the presence of silver or nitrate salts to investigate the role of these ions in the modulation of AQP1 activity.

Protein levels of *LbAQP1*-overexpressing parasites were determined by Western blot analysis. Both *L. guyanensis* and *L. braziliensis* presented at least 3.3 times increased AQP1 levels than their parental counterparts. Unlike previously described anti-AQP1 antibodies (33), the polyclonal antibodies presented here were able to recognize AQP1 in both untransfected and AQP1-overexpressing parasites, an important tool to monitor *Leishmania* AQP1 expression at the relevant protein level in resistant mutant isolates either selected in the laboratory or collected in the field.

Functional analysis showed that *L. guyanensis* and *L. braziliensis* clones overexpressing AQP1 were 1.6- and 2.6-fold more susceptible to Sb<sup>III</sup>, respectively, than the nontransfected parental lines. Gourbal et al. also observed that AQP1-overexpressing *L. tarentolae*, *L. major*, and *L. infantum* parasites presented hypersensitivity to both As<sup>III</sup> and Sb<sup>III</sup> compounds (34). These parasites exhibited higher rates of uptake of both metalloids than those transfected with the empty vector. These authors also showed that transfection of the *AQP1* gene into a sodium stibogluconate-resistant clinical isolate sensitized amastigote forms of the parasite to Sb<sup>III</sup>. On the other hand, the deletion of one allele of the *L. mexicana AQP1 (LmAQP1)* gene resulted in a mutant parasite 10-fold more resistant to Sb<sup>III</sup> (34). A dominant negative functional cloning strategy led to the isolation of a cosmid containing the *AQP1* gene, showing that AQP1 is an important route of entry of Sb<sup>III</sup> in

*Leishmania* cells (35). These authors further showed that transfection of *AQP1* increases the level of Sb<sup>III</sup> accumulation in cells. The findings presented here are in accordance with all these data obtained in studies using different *Leishmania* species, showing that transfection of *AQP1* leads to Sb<sup>III</sup> sensitization.

Since *LbAQP1* is harbored on polyploid chromosome 31 (36), it was thought to be essential for *Leishmania* spp. (37). However, we have recently identified an Sb-resistant *L. guyanensis* strain from which a locus containing *LgAQP1* was deleted during an *in vitro* stepwise drug selection process (16). Additionally, *LmAQP1* was successfully disrupted in *L. major* null mutants (38). These observations discourage a focus on AQP1 as a drug target; however, further studies are needed to address the development of rational strategies.

Silver is a metal that presents low toxicity for mammalian cells, and silver salts, such as silver nitrate and silver sulfadiazine, present antimicrobial activity (39, 40). When *Staphylococcus aureus* and *Escherichia coli* are exposed to silver nitrate, the Ag<sup>+</sup> ions activate the stress response and DNA damage, preventing cell replication and causing disruption of the cell wall (39). Our results revealed that silver salts (silver nitrate, silver sulfadiazine, and silver acetate) are highly toxic against *Leishmania* parasites, showing EC<sub>50</sub>s of 1 to 3  $\mu$ M. Under cell culture medium conditions, AgNO<sub>3</sub> dissociation is favored by the surrounding NaCl and proteins, where Ag<sup>+</sup> forms colloidal dispersed AgCl nanocomplexes related to cell toxicity (41).

Silver compounds were previously described to be potential inhibitors of aquaporins from plants and humans (22). In order to check the inhibitory potential of silver and nitrate ions, we performed competition assays on *L. braziliensis* and *L. guyanensis* lines overexpressing AQP1. Interestingly, our results showed a reduced sensitivity to Sb<sup>III</sup> upon cotreatment with Sb<sup>III</sup> and silver nitrate, Sb<sup>III</sup> and silver sulfadiazine, or Sb<sup>III</sup> and silver acetate. Metal ions, such as gold and silver ions, were described to be potent inhibitors of water transport through AQPs. Silver reacts with the sulfhydryl group from a motif conserved near the NPA (asparagine-proline-alanine) cysteine of the AQPs and then effectively blocks the region of channel constriction (22). The reduced Sb<sup>III</sup> sensitivity phenotype that emerged upon silver treatment is compatible with the suggestion that the metal inhibits AQP1, since lines that overexpressed AQP1 presented pronounced differences in sensitivity from that of lines that did not. This is the first report showing evidence of the modulation of the drug susceptibility mediated by AQP1 in *Leishmania* by silver and Sb<sup>III</sup> in combination. Thus, modulation of the drug susceptibility phenotype by silver can be a pharmacological tool to study AQP1-mediated drug resistance in *Leishmania* parasites.

Competition assays revealed that cotreatment with sodium or potassium nitrate and Sb<sup>III</sup> enhanced the susceptibility of *Leishmania* parasites to Sb<sup>III</sup>, especially AQP1-overexpressing mutants. Our results also showed that nitrate compounds presented very low toxicity against *Leishmania* and had high EC<sub>50</sub>s (120 to 160  $\mu$ M). To further investigate this antileishmanial activity, we measured the amount of antimony accumulated in the *L. guyanensis* wild-type parasites incubated in the presence of Sb<sup>III</sup> and silver or nitrate salts. The data showed high intracellular antimony concentrations in parasites incubated with Sb<sup>III</sup> and sodium or potassium nitrate. Interestingly, the results showed that antimony-mediated antileishmanial activity is enhanced when antimony is combined with a nitrate source. Indeed Sb-nitrate cotreatment favors intra-

cellular antimony accumulation, increasing drug toxicity and leading to parasite death. We checked by electrospray ionization-mass spectrometry that potassium antimonyl tartrate, used as a source of Sb<sup>III</sup>, did not form any new chemical species when mixed with potassium or sodium nitrate (data not shown). Thus, our data taken altogether indicate an additional effect of nitrate on Sb<sup>III</sup> activity, explaining the expression of antileishmanial activity, and suggest the possible modulation of AQP1 activity by nitrate.

Studies indicated that AQP6 may function as a nitrate channel in mammalian cells (42). However, unlike other aquaporins, AQP6 is permeated by ions. AQP1 from *Leishmania*, the closest homologue of human AQP9, is permeated by water or small uncharged solutes, and nitrate ions probably do not pass through AQP1. In aqueous solution at physiological pH, trivalent antimoniate (Sb<sup>III</sup>) exists mainly in the trihydroxylated uncharged form, Sb(OH)<sub>3</sub>, which structurally resembles glycerol (12). Thus, the major entry route of Sb<sup>III</sup> in *Leishmania* parasites is through AQP1, which water and glycerol are able to permeate. How nitrate may modulate the activity of AQP1 requires further evaluation. It has been suggested that Sb<sup>III</sup> can also enter cells via hexose transporters (43), and nitrate could perhaps facilitate Sb<sup>III</sup> entry into the cell using this route.

The findings presented here show that Sb<sup>III</sup>-resistant *L. guyanensis* parasites are also susceptible to treatment with the Sb<sup>III</sup>-sodium nitrate and Sb<sup>III</sup>-potassium nitrate combinations. This result indicates that this cotreatment also affects the mechanisms of Sb<sup>III</sup> resistance, as the Sb<sup>III</sup>-resistant parasite is resensitized. Previous results demonstrated that this Sb<sup>III</sup>-resistant *L. guyanensis* line presents a decrease in AQP1 expression and reduced antimony accumulation (30). Interestingly, both of these Sb<sup>III</sup> and nitrate combinations are very toxic for these resistant parasites. These results suggesting the potential of combined treatment with nitrate salts and Sb<sup>III</sup> to have antileishmanial activity are very promising and should be further explored.

Our findings showed that overexpression of the *AQP1* gene increased the sensitivity of *L. braziliensis* and *L. guyanensis* to Sb<sup>III</sup>. In addition, the present study suggests for the first time the role of silver as a putative AQP1 inhibitor in *Leishmania*, reducing Sb<sup>III</sup> entry into the cell and thereby making it more resistant to Sb<sup>III</sup>. Interestingly, treatment with nitrate salts and Sb<sup>III</sup> favors the accumulation of antimony into the parasite and increases the toxicity of the drug, culminating with parasite death. These data support the rational drug development approaches urgently needed to overcome resistance.

## ACKNOWLEDGMENTS

We thank Stephen Beverley for kindly providing the pIR1-BSD expression vector and the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for the use of its facilities.

## FUNDING INFORMATION

This study received financial support from the following agencies: CNPq/Universal (475782/2012-7), CNPq/BJT (401936/2013-9), CNPq/Pq (304483/2015-0), the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG-CBB-PPM00196/13 and 00610/15), UNICEF/UNDP/World Bank/WHO (TDR), and the P3D-Programa de Descoberta e Desenvolvimento de Drogas (PROEP/CNPq/FIOCRUZ 401988/2012-0). R.L.M.-N. and S.M.F.M. are researchers supported by CNPq-Brasil (National Council for the Scientific and Technological Development). J.M.A. is supported by a doctoral fellowship from

CAPES (Coordination for the Improvement of Higher Education Personnel). R.L.M.-N. holds a young talent fellowship (CNPq/BJT-A 314024/2013-1).

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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