Role of Aquaglyceroporin (AQP1) Gene and Drug Uptake in Antimony-resistant Clinical Isolates of Leishmania donovani

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Abstract. Antimonial-containing drugs are the first line of treatment against Leishmaniasis. Resistance to antimonials in Leishmania is proposed to be due to reduced uptake of trivalent antimony (SbIII) through the aquaglyceroporin (AQP1). We investigated the uptake of SbIII and involvement of aquaglyceroporin in developing antimony resistance phenotype in Leishmania donovani clinical isolates. SbIII accumulation, copy number of AQP1 gene, and transcript levels were compared in antimony-sensitive versus -resistant isolates. Antimony-resistant field isolates showed reduced uptake of SbIII. The copy number of AQP1 gene showed higher copy number in the antimony-resistant isolates when compared with the sensitive isolates and did not correlate to the reduced uptake of SbIII. Downregulation of AQP1 RNA levels was not consistently found in the antimony-resistant isolates. Our studies indicate that while downregulation of AQP1 may be one of the mechanisms of antimony resistance, it is however not an invariable feature.

INTRODUCTION

Visceral leishmaniasis (VL) is a protozoan parasitic disease caused by Leishmania donovani and is often fatal if left untreated. Pentavalent antimonials such as sodium antimony gluconate (SAG) are the standard first-line choice against the disease.1 Although pentavalent antimonials are age-old conventional therapy, the mechanism of action of SAG is not precisely defined. General agreement is that pentavalent form (SbV) is reduced to the more toxic trivalent form (SbIII).2 Trivalent form of the antimony constitutes the active form of drug against the parasite. The reduction of the metal may take place either in the parasite3-6 or in the macrophages7 or in both. Resistance to SAG is increasing and has been reported in several parts of the world.1,8 In the endemic regions, resistance to class of drugs is a major impediment to treatment.8 It has been demonstrated that more than 60% of the patients do not respond to SbV treatment in the Bihar state of northern India.8-10 The mechanism of action and resistance to SAG in the field isolates is less well defined. A consistent resistance mechanism deduced from in vitro work involves multiple steps and implicates trypanothione metabolism and drug transport.11-14

The ABC transporter, MRPA, has been shown to confer resistance to antimonials by sequestering metal-trypanothione conjugates into an intracellular organelle.15 Our earlier work on the clinical kala azar L. donovani isolates from India showed MRPA overexpression as an important SAG resistance factor.16 Role of a metal-thiol efflux pump in antimony resistance has also been reported but the nature of this pump is not known.17 Yet another mechanism leading to downregulation of an uptake system is the loss of an aquaglyceroporin (AQP1) allele that has been reported to cause an increase in resistance to SAG.18,19

Aquaglyceroporins (AQP1s) are the members of the aquaporin super family. They are membrane channels that permit transport of small neutral solutes such as glycerol or urea.20 Glycerol Facilitator (GlpF) and Glycerol Channel Protein (Fps1p), an aquaglyceroporin family member, have been reported to transport SbIII in Escherichia coli21 and in Saccharomyces cerevisiae,22 respectively. In Leishmania species, AQP1 has been shown to facilitate SbIII transport.18 Increased rates of uptake of SbIII correlated with the antimony sensitivity of the wild-type and drug-resistant transfectants of Leishmania18,19 suggesting AQP1 as the major route of entry of trivalent antimony in laboratory conditions. In laboratory-raised SbIII-resistant Leishmania strains, gene expression experiments have shown that AQP1 transcript levels correlated well with the accumulation of SbIII and resistance levels in Leishmania.19

In the present study we examined the correlation between the SAG sensitivity profiles of Indian field isolates of L. donovani collected from SAG responsive and unresponsive patients with SbIII accumulation. The status of AQP1 gene both at genetic and transcriptional level and the correlation with SAG resistance has been reported.

MATERIALS AND METHODS

Parasite and culture condition. Promastigotes of Leishmania donovani strains AG83 (MHOM/IN/80/AG83), GE1 (MHOM/IN/80/GE1F8R) along with four untyped strains 2001, 41, NS2, and CK2 were isolated from patients with VL and were routinely cultured at 22°C in modified M-199 medium (Sigma, USA) supplemented with 100 U/mL penicillin (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA), and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Life Technologies Scotland, UK). Clinical isolates obtained from VL patients who responded to SAG chemotherapy were designated as SAG-S (SAG-sensitive) whereas isolates from VL patients who did not respond to SAG were designated as SAG-R (SAG-resistant). Accordingly, SAG-S isolates used in this study include AG83-S and 2001-S whereas the four SAG-R isolates were 41-R, GE1-R, NS2-R, and CK2-R. These isolates have been characterized earlier.16 The SAG-resistant isolates were maintained in the absence of drug pressure in vitro. The isolates have been passaged through hamsters or BALB/c mice to retain their virulence and importantly, their chemosensitivity profiles have remained unchanged as measured periodically by amastigote-macrophage infectivity assay reported earlier.16

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Chemosensitivity profiles of SAG-S and SAG-R promastigotes to potassium antimony tartrate (SbIII). Briefly, (1 × 10^5 cells/200 µL/well) log phase promastigotes of SAG-S and SAG-R isolates were plated in triplicates in M199 medium supplemented with 10% FBS in 96-well culture plate. After 24 h of incubation at 22°C, SbIII was added in increasing concentrations (0–100 µM). After 72 h of drug exposure, live cells were counted using a hemocytometer. The 50% inhibitory concentration (IC_{50}) was extrapolated from the graph representing different concentrations of SbIII plotted against the percentage of viable cells.

**Uptake assay.** Uptake studies were done as described previously. Briefly, log phase *Leishmania* promastigotes were washed twice with phosphate buffered saline (PBS, pH 7.4) and were resuspended in PBS containing 10 mM glucose at a density of 5 × 10^7 cells/mL. Cells were then incubated at 27°C with 100 µM SbIII for 30 minutes and an aliquot of 500 µL samples were collected at 0 and 30 minutes. After that cells were pelleted down and washed twice with equal volume of ice cold PBS, centrifuged at 17,000 g for 1 minute at room temperature, dried and digested with 0.05% of 70% nitric acid for 2 h at 70°C, and then diluted with 3 mL of the high-pressure liquid chromatography grade water. Antimony levels were analyzed by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) as reported earlier. Each uptake assay was repeated twice with duplicates in each set.

**Cloning of full-length aquaglyceroporin (AQP1) gene from antimony-sensitive and antimony-resistant *L. donovani* strains.** To clone the gene encoding aquaglyceroporin (AQP1) from *L. donovani*-sensitive and -resistant strains, PCR was performed using specific oligonucleotides, whose sequence was based on *Leishmania* Genome Sequencing Project of *Leishmania infantum* (www.ebi.ac.uk/parasites/LGN/). DNA fragments of 945 bp were amplified from genomic DNA of 2001-S and GE1-R strains, using a sense primer with a flanking *Xba*I site, 5'-GC TCTAGA ATGAACTCTC CTACAAGCAC A-3’ which coded for the amino acid sequence MNSTST at position 1-21, and the antisense primer with a flanking *Hind*III site, 5’-CCC AAGCCT CTAGAAGTTGGGTAGAATGA-3’, which corresponded to amino acid residues IIPPNF including the stop codon, at position 926-945. Polymerase chain reaction (PCR) was performed in a 50 µL reaction volume containing 100 ng of genomic DNA, 200 pmol each of gene-specific forward and reverse primers, 200 µM of each dNTP, 2 mM MgCl2, and 5 U *Taq* DNA polymerase (MBI Fermentas). The condition of PCR was as follows: 94°C for 10 min, 94°C for 45 s, 60°C for 30 s, 72°C for 45 s, and 35 cycles. Final extension was carried for 10 min at 72°C. Single band of 945-bp PCR products were obtained and subcloned in to pGEM-T vector (Promega) and subjected to automated sequencing. Sequence analysis was performed by DNASTar whereas comparison with both sequences and with other sequences were performed using the search algorithm BLAST. Multiple alignment of amino acid sequences was performed using CLUSTAL W program.

**Nucleic acid isolation and hybridization analysis.** Genomic DNA was isolated from ~2 × 10^6 promastigotes (AG83–S, 2001–S, GE1–R, 41–R, CK2–R, and NS2–R) by standard procedure. Genomic DNA were digested with Sall or XhoI restriction enzymes and subjected to electrophoresis in 0.8% agarose gel. The fragments were transferred to Hybond™- N+ membrane (Amersham Pharmacia Biotech) and subjected to Southern blot analysis. Total RNA was isolated from ~2 × 10^6 promastigotes (AG83–S, 2001–S, GE1–R, 41–R, CK2–R, and NS2–R) using TRI Reagent™ (Sigma). For Northern blot analysis, 15 µg of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred onto Hybond™- N+ membrane following standard procedures. Following the transfer of DNA and RNA onto nylon membranes, the nucleic acids were cross-linked to the membrane in UV cross linker. Prehybridization was done at 65°C for 4 h in a buffer containing 0.5 M sodium phosphate; 7% SDS; 1 mM EDTA, pH 8.0, and 100 µg/mL sheared denatured salmon sperm DNA. The blots were hybridized with denatured [α-32P] dCTP- labeled DNA probe at 10°C cm/mL, which was labeled by random priming (NEB Blot kit, New England Biolabs). The full length (945 bp) *Leishmania dono- vanii* aquaglyceroporin gene (AQP1) (genebank accession number, EF600686) from the strain 2001-S was used as probe. Membranes were washed, air dried, and exposed to imaging plate. The image was developed by phosphor Imager (Fuji film FLA-500, Japan) and RNA levels were quantified using Image quant software.

**RESULTS**

**Susceptibility of *Leishmania* isolates to potassium antimony tartrate (SbIII).** SAG sensitivity profiles of *Leishmania* isolates (AG83–S, 2001–S, GE1–R, 41–R, CK2–R, and NS2–R) has been reported earlier using amastigote-macrophage model system and the IC_{50} values for AG83–S and 2001–S were 9 ± 0.5 µg/mL and 13 ± 1.5 µg/mL, respectively while that of the resistant field isolates GE1–R, 41–R, CK2–R, and NS2–R were > 100 µg/mL, 65 ± 3.4 µg/mL, 55 ± 2.5 µg/mL and 24 ± 1.4 µg/mL respectively to SAG. While the drug is being used as pentavalent antimonial compound (SbV), the active form of the drug is a trivalent antimony (SbIII). Previous studies have shown a correlation between resistance to SbIII in both promastigotes and intracellular amastigotes. We therefore used promastigotes to check the effect of SbIII on the parasites and have used promastigotes in the present work for further characterization of SbIII transport. The IC_{50} value to SbIII of SAG-sensitive promastigotes, AG83–S and 2001–S were 15 ± 3.5 µM and 8 ± 2.1 µM respectively, whereas the IC_{50} value of SAG-resistant field isolates GE1–R, 41–R, CK2–R, and NS2–R were 23 ± 1.8 µM, 25 ± 3.2 µM, 22 ± 1.9 µM, and 38 ± 2.2 µM, respectively (Table 1). The results indicated that 2001–S is most susceptible towards SbIII when compared with the rest of the isolates. SAG-resistant isolates had higher IC_{50} value with SbIII when compared with the sensitive isolates AG83–S and 2001–S. The resistant iso-

**TABLE 1**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Mean IC_{50} ± SD (µM)</th>
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<tbody>
<tr>
<td>AG83–S</td>
<td>15 ± 3.5</td>
</tr>
<tr>
<td>2001–S</td>
<td>8 ± 2.1</td>
</tr>
<tr>
<td>GE1–R</td>
<td>23 ± 1.8</td>
</tr>
<tr>
<td>41–R</td>
<td>25 ± 3.2</td>
</tr>
<tr>
<td>CK2–R</td>
<td>22 ± 1.9</td>
</tr>
<tr>
<td>NS2–R</td>
<td>38 ± 2.2</td>
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IC_{50} were determined in promastigotes after 72 h of drug addition. The results are mean ± SD of three independent experiments.
lates GE1-R, NS2-R, 41-R, and CK2-R were ~3–5-fold more resistant to SbIII compared with the sensitive isolate.

Comparison of transport of potassium antimony tartrate (SbIII) in SAG-R versus SAG-S field isolates. The effect of a drug depends on the amount of drug transport and accumulation inside the cells. The reduced uptake of the drug could be a mechanism by which cells develop resistance to antimonials. Lack of commercial source of 125Sb has made direct measurement of Sb accumulation in Leishmania complex. In the present study, we have used Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) to show SbIII accumulation inside the cells. We first compared the accumulation of SbIII in a SAG-S and a SAG-R L. donovani as measured by ICP-MS. Promastigotes were incubated with 100 μM SbIII at 27°C for 30 min, centrifuged, washed three times with PBSG, and resuspended in drug-free PBSG. Retained SbIII was measured between 0 and 30 min. Results are mean ± SD of two replicate experiments.

At zero time point SAG-sensitive and -resistant field isolates showed < 10 nmol, SbIII accumulation within the cells. It was found that the SAG-R isolates had reduced accumulation of SbIII when compared with the SAG-S field isolates (Figure 2). In comparison to 2001-S, SAG-R field isolates GE1-R, 41-R, CK2-R, and NS2-R accumulated ~4.5, 4.9, 3.3,
and 6.8-fold less SbIII respectively (Figure 2). These transport studies suggest that the SAG-R *Leishmania* isolates had reduced accumulation of SbIII when compared with the SAG-S isolates.

**Southern blot analysis of the aquaglyceroporin (AQP1) gene in SAG-S and SAG-R field isolates.** Southern blot analysis clearly depicted that in *L. donovani* field isolates AQP1 gene showed variation in the gene copy number between SAG-S and SAG-R isolates. Restriction enzyme SalI, which cuts outside the gene producing 10.8 kb restriction fragment (expected size) and XhoI, which cuts once inside the gene producing 2.3 kb fragment (expected size) was used to check the copy number of the gene. SalI digestion produced a single hybridizing fragment of 10.8 kb that was observed in both SAG-S isolate 2001-S and SAG-R isolates. However, SAG-R strains, GE1-R and 41-R produced more than one low molecular weight hybridizing fragments in addition to 10.8 kb band of expected size (Figure 3A), indicating that AQP1 exist as a multiple copy gene in these two strains. Southern blot analysis of XhoI digested genomic DNA showed that the copy number of the AQP1 gene (expected band size of 2.3 kb) in SAG-R isolates was higher than that of the SAG-S isolates (Figure 3B).

Because the analysis of the Southern blot with AQP1 gene probe in SAG-S and SAG-R field isolates showed genetic polymorphism among the isolates we decided to clone the AQP1 gene from a SAG-S isolate, 2001-S, and a SAG-R isolate, GE1-R (which showed maximum resistance and genetic polymorphism). The AQP1 gene from the two strains was sequenced and the predicted protein was compared. The predicted protein of the SAG-S isolate, 2001-S (genebank accession number, EF600686) was similar to that of the SAG-R isolate, GE1-R (genebank accession number, EU191226). *L. donovani* AQP1 protein sequences obtained here showed 99%, 86%, and 82% homology to *L. infantum* (XP_001467265), *L. major* (CAJ08141), and *L. mexicana* (AAW56828) respectively (Figure 4).

**Comparison of aquaglyceroporin gene expression in SAG-S versus SAG-R field isolates.** We studied the expression of the AQP1 gene by Northern blot analysis. In SAG-R field isolates, 41-R, CK2-R, and NS2-R, the transcript levels of AQP1 were found to be down regulated when compared with the SAG-sensitive isolate, 2001-S (Figure 5). Densitometric scanning of AQP1 transcript level showed that SAG-resistant field isolates, 41-R, CK2-R, and NS2-R showed downregulation of transcript level by ~1.7-, 3.7-, and 1.5-fold respectively in comparison to the SAG-S isolate, 2001-S (Figure 5). Surprisingly, the strain GE1-R, which had been shown earlier to be most resistant to SAG in comparison to all other resistant isolates, had transcript levels that were comparable to that of the SAG-S isolate, 2001-S. Low transcript levels of AQP1 were observed in AG83-S isolate when compared with another SAG-S isolate, 2001-S.

**DISCUSSION**

Sodium antimony stibogluconate (SAG) is the drug of choice against *Leishmania* and resistance to this drug is a major problem in the field not only in the Indian subcontinent, but also throughout the world. This increase in resistance to SAG has led to an upsurge in therapeutic failure, and in the absence of limited chemotherapeutic alternatives, it is extremely relevant that mechanisms of resistance be evaluated in field isolates.

Decreased uptake of the drug is one of the mechanisms by which resistance can occur. In case of *Leishmania* lesser accumulation of antimony could lead to resistance phenotype.24 It has been reported earlier that AQP1 is an important transporter by which SbIII can accumulate within *Leishmania* cells but may not be the only one.18,19,24 AQP1 modulates drug sensitivity when expressed at increased levels in both promastigotes and amastigotes.18 In *Leishmania* spp. transfection of AQP1 restored SbIII transport and regained susceptibility to antimony in resistant cells.18,19 It has been reported earlier that reduced accumulation of SbIII in SbIII-resistant mutants
could be due to decreased activity of ShIII channel, AoPI.\textsuperscript{18,19} Loss of AoPI has been shown to produce resistance and increased expression of AoPI in drug-resistant parasites reversed resistance.\textsuperscript{18}

Our earlier studies using SAG-S and SAG-R clinical isolates showed susceptibility to SAG as determined \textit{in vitro} with intracellular amastigotes correlated well with the clinical response.\textsuperscript{16} The ABC transporter gene \textit{MRPA} was amplified in resistant field isolates as part of an extrachromosomal circle.\textsuperscript{16} Amplification of \textit{MRPA} was correlated to increased RNA as determined by real-time PCR.\textsuperscript{16}

In the present study, we looked at the involvement of aquaglyceroporin in developing SAG-resistance phenotype in these \textit{L. donovani} field isolates that have been characterized earlier.\textsuperscript{16} To test this we monitored the uptake of ShIII by SAG-S and SAG-R isolates. Reduced uptake of the drug in SAG-R isolates was observed when compared with the uptake in SAG-S isolates. Thus a correlation was observed between the levels of ShIII accumulation and sensitivity profiles of the isolates. Uptake of ShIII in SAG-R field isolates correlated well with the earlier findings using the laboratory-raised, antimony-resistant strains\textsuperscript{19} indicating a similar mechanism could also be operative in the field isolates. The decreased accumulation could be due to decreased uptake possibly due to a point mutation in the putative channel or increased efflux of the metal. The efflux results indicate that the initial rate of efflux of ShIII in sensitive and resistant lines is similar. There are two possible explanations of the observed similar initial rates of efflux in the sensitive and resistant lines. In the first case it is assumed that a protein involved in efflux is found in similar levels in both lines and that the available ShIII concentrations in both lines are high enough to saturate the efflux capacity. According to this model, however, the fact that in the sensitive
line the rate of efflux does diminish in time, and retained drug remains ~6-fold higher than those enabling rapid efflux from the resistant cell line, would indicate that the sensitive line was able to sequester more antimony than the resistant line was able to sequester.

An alternative explanation could also account for similar absolute rates of elimination in the initial phase. In this case, because the resistant line has around 3-fold less internalized drug at the start of the measured period, it could be that the resistant line has a 3-fold higher level of efflux activity enabling it to excrete at a similar rate to the sensitive line. This would assume that the quantity of substrate available for efflux falls well beneath the saturating level of carrier for substrate. To differentiate between these processes a more detailed characterization of the kinetic parameters of the carrier responsible for efflux would be required. Earlier observations have reported no difference in the efflux of SbIII between the sensitive and the resistant cells.19

We also monitored the copy number of AQP1 gene and the RNA expression levels in SAG-S and SAG-R isolates. The copy number of the AQP1 gene was higher in SAG-R isolates in comparison to the SAG-S isolates (Figure 3B) and does not correlate with the reduced SbIII uptake in SAG-R isolates observed in the present study. This data differs from the earlier findings using laboratory-raised mutants where the copy number of AQP1 remained unchanged for all the resistant mutants.19 Because Southern blot analysis of AQP1 gene showed genetic polymorphism in the field isolates we cloned AQP1 gene from one SAG-S isolate and one SAG-R isolate. Our results indicate that at least in GE1-R, reduced accumulation observed does not result from point mutation in the coding sequence of AQP1 gene.

Previous studies have shown downregulation of AQP1 gene expression in SbIII-resistant mutants selected in the laboratory and the levels of transcripts correlated with the concentration of SbIII used for selecting the resistant mutants and also with the reduced metal accumulation.19 Northern blot analysis of the clinical isolates in the present study showed downregulation in SAG-R isolates; 41-R, CK2-R, and NS2-R when compared with 2001-S, a SAG-S strain. However, GE1-R, which had been reported earlier to be the most resistant of all the isolates (IC_{50} > 100 μg/mL)16 showed higher gene copy number and upregulation of AQP1 RNA levels when compared with other resistant isolates and RNA levels were more than that of the SAG-S isolate, 2001-S. Increased level of AQP1 transcript in GE1-R isolate and decreased RNA levels in AG83-S isolate indicates that besides AQP1 other mechanisms of antimonial resistance are operative. Our studies indicate that while downregulation of AQP1 may be one of the mechanisms of antimony resistance it is however not an invariable feature of such resistance. Though our studies represent limited set of clinical isolates, however, they do differ significantly from results obtained in selected laboratory strains where AQP1 has been shown to be a key determinant of antimony accumulation and susceptibility in _L. donovani_.16 Similar exceptions were observed earlier in amastigotes of _L. infantum_ strain selected for resistance to SbIII.19 It is also possible that an alternative pathway of SbIII uptake different from AQP1 may exist in the field isolates.

Taken together this study suggested the role of AQP1 gene in generation of antimony-resistant phenotype to be important, but not a sole determinant of antimony resistance in the Indian field isolates. Further studies with more number of isolates are needed to address this question.

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**FIGURE 5. Expression of aquaglyceroporin at transcriptional level in SAG-S and SAG-R field isolates of _L. donovani_. (A) Total RNA was isolated from promastigotes, electrophoresed, blotted, and hybridized with AQP1 specific probe of 945 bp (B) RNA amount layered on the gel was monitored by rehybridizing the blots with α-tubulin probe. Hybridization signals were quantified relative to 2001–S and are included within square brackets. Relative transcriptional upregulation and downregulation were indicated by (+) and (−) before numerical value. Lane 1: AG83-S, Lane 2: 2001-S, Lane 3: GE1-R, Lane 4: 41-R, Lane 5: CK2-R, Lane 6: NS2-R.**

**REFERENCES**


6. Zhou Y, Messier N, Ouellette M, Rosen BP, Mukhopadhyay R,


