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

Mahendra Maharjan, Sushma Singh, Mitali Chatterjee, and Rentala Madhubala*

School of Life Sciences, Jawaharlal Nehru University, New Delhi, India; Department of Pharmacology, Institute of Postgraduate Medical Education and Research, Kolkata, India

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 parasite[3–6] or in the macrophages[7] 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 this 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 coli*[21] 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 *Leishmania*[18,19] 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]

* Address correspondence to Rentala Madhubala, School of Life Sciences, Jawaharlal Nehru University, New Delhi – 110067 India. E-mail: madhubala@mail.jnu.ac.in

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.[19] 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.[3,19] 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 MNSPTST at position 1-21, and the antisense primer with a flanking *Hin*dIII site, 5′-CCC AAGCTT CTAGAAGTTGGGTGGAATGA-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, 25 pmol each of gene-specific forward and reverse primers, 200 μM of each dNTP, 2 mM $MgCl_2$, 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 ~2×10^8 promastigotes (AG83–S, 2001–S, GE1–R, 41–R, CK2–R, and NS2–R) by standard procedure.[23] Genomic DNA were digested with SalI 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 ~2×10^8 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-^{32}P] dCTP- labeled DNA probe at 10^6 cpm/mL, which was labeled by random priming (NEB Blot kit, New England Biolabs). The full length (945 bp) *Leishmania donovani* 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.[16] While the drug is being used as pentavalent antimonial compound (SbV), the active form of the drug is a trivalent antimony (SbIII).[3] Previous studies have shown a correlation between resistance to SbIII in both promastigotes and intracellular amastigotes.[18,19] 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

IC_{50} of promastigotes of SAG-sensitive and SAG-resistant *L. donovani* field isolates to SbIII

Isolates	Mean IC_{50} ± SD (μM)
AG83-S	15 ± 3.5
2001-S	8 ± 2.1
GE1-R	23 ± 1.8
41-R	25 ± 3.2
CK2-R	22 ± 1.9
NS2-R	38 ± 2.2

IC_{50}s were determined in promastigotes after 72 h of drug addition. The results are mean ± SD of three independent experiments.

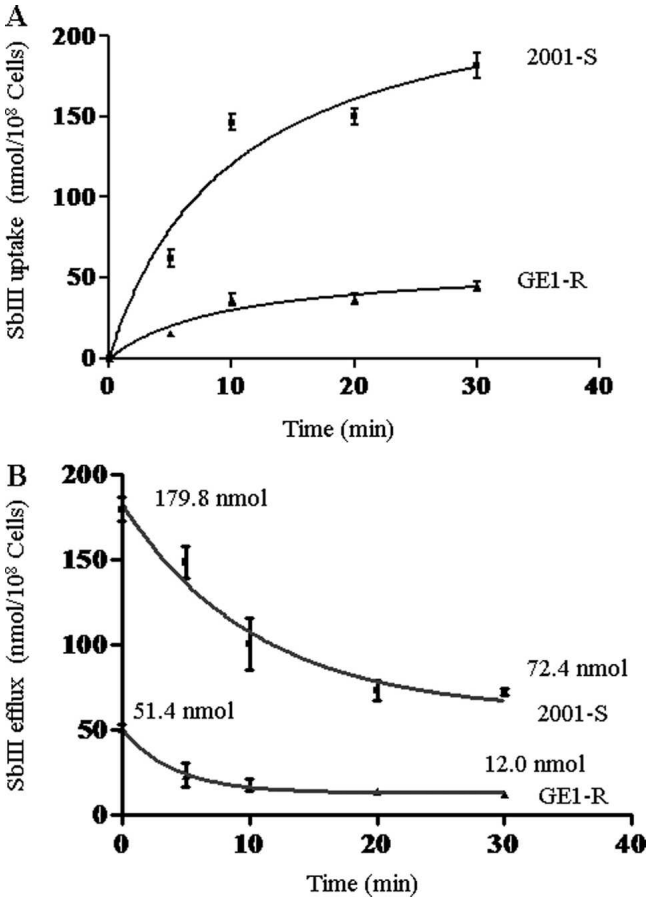

FIGURE 1. **A**, Time-dependent accumulation of SbIII in SAG-S and SAG-R *Leishmania donovani* as measured by ICP-MS. Promastigotes of 2001-S (■) and GE1-R (▲) were incubated with 100 μM SbIII and samples were taken at different time points as described in the Materials and Methods. Results are the mean ± SD of two replicate experiments. **B**, Efflux of SbIII by SAG-S (■) and SAG-R (▲) *Leishmania donovani* field isolates 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.

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.[24] Lack of commercial source of ^{125}Sb 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.[3,19] We first compared the accumulation of SbIII in a SAG-S and a SAG-R *L. donovani* as a function of time. Accumulation of antimony in SAG-R isolate GE1-R showed time-dependent decrease when compared with the accumulation in SAG-S isolate, 2001-S (Figure 1A). We used 10 μM and 100 μM concentrations of SbIII to compare the dose-dependent accumulation of SbIII in a SAG-S isolate, 2001-S and a SAG-R isolate, GE1-R. The accumulation of SbIII after 30 min was 7.0 ± 3.1 nmol/10^8 cells and 1.4 ± 0.3 nmol/10^8 cells in 2001-S and GE1-R respectively, when 10 μM concentration of SbIII was used. However, when 100 μM concentration of SbIII was used the accumulation was 182 ± 11.0 nmol/10^8 cells and 44.4 ± 3.9 nmol/10^8 cells in 2001-S and GE1-R respectively (data not shown).

Efflux of SbIII from sensitive and resistant cells was compared (Figure 1B). Both lines were loaded in the same fashion (incubating with 100 μM SbIII at 27°C for 30 min). After this cells were washed and transferred to medium without SbIII for 30 min. The resistant cell line, GE1-R, accumulated markedly less SbIII over the loading period when compared with the sensitive cells, 2001-S (51 ± 3.1 nmol per 10^8 cells compared with 179.8 ± 9.8 nmol per 10^8 cells). The rate of efflux over an initial phase (the first 5-min interval) occurred at a similar rate in both cell lines (31 nmol per 10^8 cells in 2001-S, over the first 5 min and 28 nmol per 10^8 cells in the resistant line, GE1-R). Hereafter the rate of efflux tailed off quickly in the resistant line approaching a plateau by 30 min of about 12.0 ± 0.6 nmol per 10^8 cells. In the sensitive line the rapid rate of efflux continued for longer period than in the resistant line although it also approached a plateau by the 30-min point indicating about 72.4 ± 2.8 nmol per 10^8 cells retained in the parasites (Figure 1B).

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,

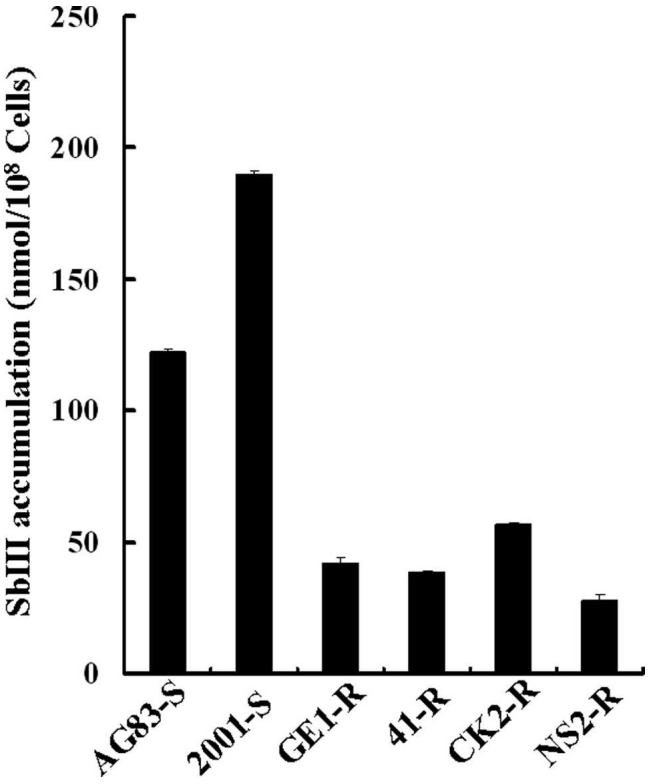

FIGURE 2. Accumulation of SbIII in SAG-S and SAG-R *Leishmania donovani* field isolates as measured by ICP-MS. Promastigotes were incubated with 100 μM SbIII for 30 minutes at 27°C as described in Materials and Methods. Data are shown as the mean ± SD of two replicate experiments.

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[16] 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.

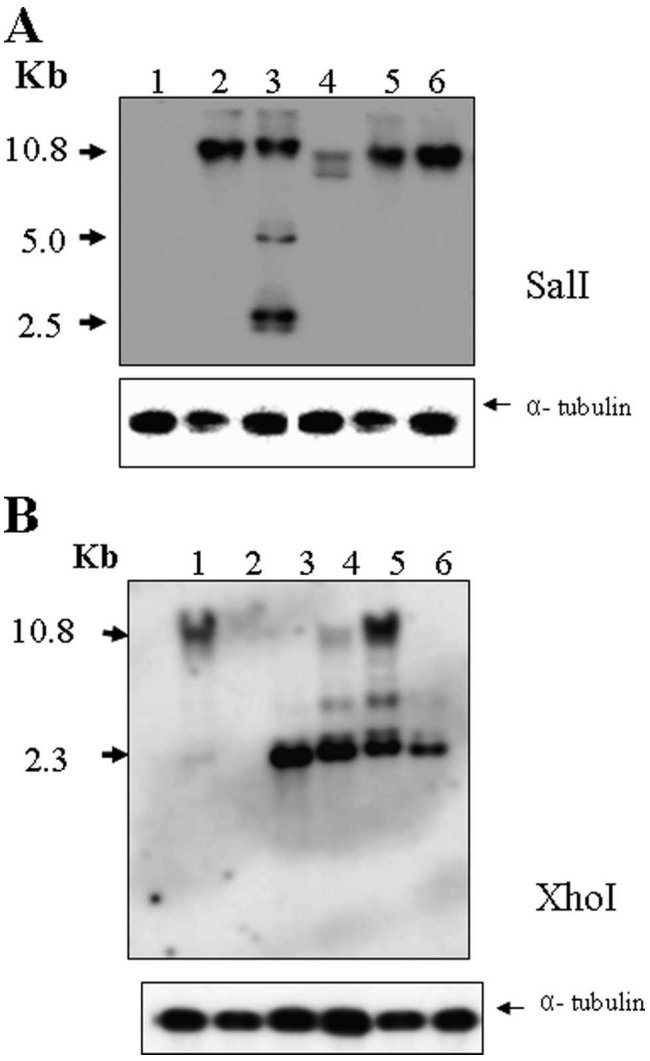

FIGURE 3. Southern blot analysis of the aquaglyceroporin gene in SAG–S and SAG–R *Leishmania donovani* field isolates. Total genomic DNA was isolated and digested with (**A**) SalI restriction enzyme, which cuts external to the gene and (**B**) XhoI restriction enzyme, which cuts once inside the gene. The digested DNA was electrophoresed, blotted, and hybridized with an AQP1 specific probe of 945 bp. The sizes of the hybridizing bands were determined using HindIII digested λ-DNA. The blot was rehybridized with α-tubulin probe to monitor the amount of digested DNA layered on the gel. Lane 1: AG83-S, Lane 2: 2001-S, Lane 3: GE1-R, Lane 4: 41-R, Lane 5: CK2-R, Lane 6: NS2-R.

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 amstigotes.[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

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2001-S          MNSPTSTPPACYDAEVQLYMDKEDPEGVPIQNQMHEEEQGQLEGKRNFTSQNRWPLYKYR 60
GE1-R           MNSPTSTPPACYDAEVQLYMDKEDPEGVPIQNQMHEEEQGQLEGKRNFTSQNRWPLYKYR 60
L. infantum     MNSPTSTPPACYDAEVQLYMDKEDPEGVPIQNQMHEEEQGQLEGKRNFTSQNRWPLYKYR 60
L. major        MNSPTTMPLQCHDAETQLYVDKESPEGLPIGNQMHEEEEDQHESKRNFMSQNRWPLYRYR 60
L. mexicana     MNSPTSMPVPCCEADAQMCMDKEDPDGVAVEHEMHQEAHRGSERKRSFTWQGKWPFYKYR 60
                *****: *  * :*:.*: :***.*:*:.: ::**:* .   * **.*  *.:**:*:**

2001-S          WWLREYVAEFFGIFFLVIFGIGVIATTVFHAGNAASYQSNSSYMAITFGWGFGLTIGLFL 120
GE1-R           WWLREYVAEFFGIFFLVIFGIGVIATTVFHAGNAASYQSNSSYMAITFGWGFGLTIGLFL 120
L. infantum     WWLREYVAEFFGIFFLVIFGIGVIATTVFHAGNAASYQSNSSYMAITFGWGFGLTIGLFL 120
L. major        WRLREYVAEFFGIFFLVIFGIGVVATTVFHGGTIAMYQSNSSYLAITFGWAFGLAISLFL 120
L. mexicana     WLVREYVAEFFGIFFLVIFGIGVIATTVFHAGNAAVYQSNTSYMEITLGWGFGLTIGLFL 120
                * :********************:******.*.:* ****:**: **:**.***:*.***

2001-S          SMAVSGGHLNPAVTLANCVFGAFPWIKLPGYFLAQFLGGLVGAANTYGLFKSHFDDAQKA 180
GE1-R           SMAVSGGHLNPAVTLANCVFGAFPWIKLPGYFLAQFLGGLVGAANTYGLFKSHFDDAQKA 180
L. infantum     SMAVSGGHLNPAVTLANCVFGAFPWIKLPGYFLAQFLGGLVGAANTYGLFKSHFDDAQKA 180
L. major        SMAVSGGHLNPAVTLANCVFGTFPWVKLPGYFLAQFLGGFVGAANTYVLFKSHFDEAEKR 180
L. mexicana     SMAVSGGHLNPAVTLANCVFGVLPWIKLPGYFLAQFLGALAGAANTYLLFKSHFDVGQKN 180
                ********************.:**:************.:.****** ******* .:*

2001-S          LLPNETMASKYSGIFATYPNVANTYAVWSEVFNTMALMMGILAITDPRMTPAVNYKPVAI 240
GE1-R           LLPNETMASKYSGIFATYPNVANTYAVWSEVFNTMALMMGILAITDPRMTPAVNYKPVAI 240
L. infantum     LLPNETMASKYSGIFATYPNVANTYAVWSEVFNTMALMMGILAITDPRMTPAVNYKPVAI 240
L. major        LLLNETMASKYGGIFATYPNVANTYAVWSEVFNTMALMMGILAITDARMTPAVDYKPVAI 240
L. mexicana     LLPNETMASKYSGIFATYPNVANTYAAWSEVFNTMALMMGILAITDPRMTPAVNFKPVAI 240
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2001-S          GLLLFVIGITSGINSSYGLNPARDLSPRILSAMLWGSEPFTLYSYYFWIPLVAPFVGALL 300
GE1-R           GLLLFVIGITSGINSSYGLNPARDLSPRILSAMLWGSEPFTLYSYYFWIPLVAPFVGALL 300
L. infantum     GLLLFVIGITTGINSSYGLNPARDLSPRILSAMLWGSEPFTLYSYYFWIPLVAPFVGALL 300
L. major        GLLLFVIGIASGINSSYGLNPARDLSPRILSAMLWGSEPFTLHSYYFWIPLVVPFVGALF 300
L. mexicana     GLLLFVIGITSGINSAYGLNPARDFAPRVFAAMLWGSEPFTMHGFYFWIPLIAPFIGALL 300
                **********::****:*********::**:::::::::::::::.:******:.**:***:

2001-S          GMFLYVFFIIPPNF 314
GE1-R           GMFLYVFFIIPPNF 314
L. infantum     GMFLYVFFIIPPNF 314
L. major        GMFLYVFFIIPPSC 314
L. mexicana     GMFFYVFFIIPPNF 314
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FIGURE 4. Multiple sequence alignment of aquaglyceroporin protein sequences from *Leishmania donovani*; SAG-S strain, 2001-S (ABQ84980) and SAG-R strain, GE1-R (EU191226), *L. infantum* (XP_001467265), *L. major* (CAJ08141), and *L. mexicana* (AAW56828) using Bioedit Sequence Alignment editor online tool analysis program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The amino acids are numbered to the right of the respective sequences. Residues that are conserved completely are marked with (∗) and residues conserved at least in three sequences are marked with (:) below the respective sequences. *L. donovani*; 2001-S sensitive strain has 100% identity with *L. donovani* GE1-R resistant strain, 99% identity *with L. infantum,* 86% identity with *L. major*, and 82% identity with *L. mexicana*.

could be due to decreased activity of SbIII channel, AQP1.[18,19] Loss of AQP1 has been shown to produce resistance and increased expression of AQP1 in drug-resistant parasites reversed resistance.[18]

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

In the present study, we looked at the involvement of aquaglyceroporin in developing SAG-resistance phenotype in these *L. donovani* field isolates that have been characterized earlier.[16] To test this we monitored the uptake of SbIII 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 SbIII accumulation and sensitivity profiles of the isolates. Uptake of SbIII in SAG-R field isolates correlated well with the earlier findings using the laboratory-raised, antimony-resistant strains[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 SbIII 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 SbIII concentrations in both lines are high enough to saturate the efflux capacity. In this situation, similar amounts of efflux protein would result in similar rates of efflux, despite the differing levels of intracellular antimony. According to this model, however, the fact that in the sensitive

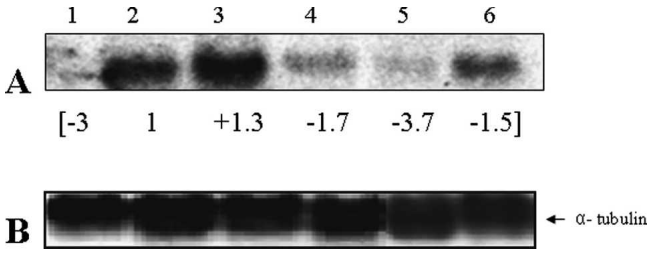

FIGURE 5. Expression of aquaglyceroporin at transcriptional level in SAG-S and SAG-R field isolates of *L. donovani.* (**A**) Total RNA was isolated from promastigotes, electrophoresed, blotted, and hybridized with AQP1 specific probe of 945 bp (**B**) RNA amount layered on the gel was monitored by rehybridizing 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.

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.[24]

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*.[19] 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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Authors' addresses: Mahendra Maharjan, Sushma Singh, and Rentala Madhubala, School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India, Tel: +91-11-26106630, Fax: 91-11-26106630, E-mails: mahendra_maharjan@yahoo.ca, sushmasingh76@yahoo.com, and madhubala@mail.jnu.ac.in. Mitali Chatterjee, Department of Pharmacology, Institute of Postgraduate Medical Education and Research, 244B Acharya JC Bose Road, Kolkata-700 02, India, Tel: +0-88-983-003-1523, E-mail: mitali4135@dataone.in.

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