Drug resistance mechanisms in clinical isolates of *Leishmania donovani*

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*Leishmania* are protozoan parasites distributed worldwide. About 1.5-2.0 million cases are reported in the world annually from this disease and the death toll is estimated to be 57,000. Along with Brazil, Sudan and Bangladesh, India contributes to 90 per cent of the global burden of visceral leishmaniasis (VL). The absence of effective vaccines and vector control programmes, makes chemotherapy the most widely used tool against leishmaniasis. Chemotherapy based on pentavalent antimonials has been used for more than 50 years and remains the mainstay for treatment of leishmaniasis. Clinical resistance to pentavalent antimonials, in the form of sodium antimony gluconate (SAG), has become a major problem in the treatment of kala-azar (visceral leishmaniasis) in India. The mechanism of resistance is unclear in these clinical isolates although a lot of work has been carried out with *Leishmania* mutants selected in vitro by step-wise increasing drug concentration using the antimony related metal arsenic and more recently sodium antimony gluconate. We for the first time, investigated the molecular aspect of drug resistance in clinically confirmed sodium antimony gluconate resistant field isolates and found that the parasite evaded cytotoxic effects of therapy by enhanced efflux of drugs through overexpressed membrane proteins belonging to the superfamily of ABC (ATP-binding cassette) transporters. Additionally, our study also points towards cell surface changes in resistant isolates.

**Key words** Clinical isolates - drug resistance - *Leishmania donovani* - molecular mechanism - sodium antimony gluconate

*Leishmania* protozoan parasite, belongs to the family of trypanosomatids and is responsible for a group of diseases whose symptoms range from mild cutaneous lesions to fatal visceral involvement. Today, the leishmaniases are endemic in 88 countries with an estimated 350 million people at risk. It has been estimated that 12 million people are affected by this group of diseases with around 1.5 to 2 million new cases occurring annually; and this number is rising¹. The group of diseases caused by *Leishmania* parasites is transmitted by the bite of sandflies. In humans, the disease occurs in four forms; life threatening visceral leishmaniasis (VL), commonly known as kala-azar; mucosal leishmaniasis (MCL), self-healing cutaneous leishmaniasis (CL), and post-kala-azar dermal leishmaniasis (PKDL). Visceral leishmaniasis is fatal, if left untreated. In 1999, there were 57,000 deaths reported in India due to VL, but the real number is thought to be significantly higher. Ninety per cent of those afflicted by VL live in five
developing countries namely, India (especially Bihar), Bangladesh, Nepal, northeastern Brazil and Sudan.

Current treatment options for patients with leishmaniasis

Pentavalent antimony, the most widely prescribed drug to treat leishmaniasis patients, was discovered a century ago, has serious side effects, requires a prolonged course of treatment and is losing its efficacy in some regions due to increasing parasite resistance. Although newer treatments exist, they are not optimal due to problems of toxicity, high price or difficulty in administration. Co-infection with HIV poses an additional challenge. In northern Bihar, resistance levels of up to 65 per cent have been recorded in parasites and other regions might also follow2,3. Research over the past decade has identified a number of drugs and formulations that offer improved treatment for this disease4,5. The main alternative currently available in the area is amphotericin B (AmB). Although highly efficacious, AmB treatment is associated with serious side-effects, and can only be given in hospital settings. Other alternatives exist to treat VL6 but these remain largely inaccessible to patients because they are too expensive (AmBisome, miltefosine which is teratogenic, only registered in India and expensive) or not registered (paromomycin). Given the problems of toxicity, need for hospitalization, growing resistance, and high costs associated with the currently available drugs for leishmaniasis, it is clear that patients urgently need new and improved treatments to replace or complement these drugs. Excellent recent reviews deal with Leishmania and anti-leishmanial drugs available or under development7,8.

Antimony treatment

It is quite remarkable that even after 50 years of clinical use, the mode of action of antimony is unknown, but there is a general belief that to be active, pentavalent form Sb(V) needs to be reduced to the trivalent form9. Evidence for reduction inside the parasites has been described10. An alternative view is that the metal is reduced in the macrophage of the host11. Reduction could either occur enzymatically, as in yeast12, or by parasite or host derived thiols13. Parasite-specific thiols such as trypanothione as well as macrophage-specific thiols such as glycerylcysteine, can reduce SbV to SbIII non enzymatically. Recently, a parasite-specific enzyme thiol dependent reductase (TDR1), that contains domains with similarities to omega glutathione transferases, was shown to catalyze the conversion of SbV to SbIII using glutathione as a reductant14. A new antimoniate reductase, ACR2, was characterized in Leishmania and was shown to reduce SbV and to increase the sensitivity of Leishmania cells to SbV15. Recent data suggest that antimony compromises the thiol redox potential of the cell by inducing the efflux of intracellular thiols and by inhibiting trypanothione reductase16. It is possible that more than one mechanism are responsible for drug activation. Antimonials are thought to act directly by targeting important biological features of the parasite. In macrophage infection models, SbV is leishmanicidal, but in an animal infection model its mode of action is dependent on a number of factors including T cell subsets and cytokines17. Stibogluconate was found to be a potent inhibitor of protein tyrosine phosphatases, leading to an increase in cytokine responses18. These results suggest that SbV may kill the parasites by both direct and indirect mechanisms, the host response being implicated in the activity of SbV. It has been shown that both SbIII and SbV mediate DNA fragmentation in Leishmania species, suggesting that antimony kills the parasite by a process reminiscent of apoptosis19,20. The routes of entry of antimonials into Leishmania (or into macrophages) are not known, although pentavalent arsenate, a metal related to SbV, is known to enter via phosphate transporters21. The accumulation of SbV is measured with radioactive isotopes22, and lately using mass spectrometric approaches23.

Potential mechanisms of drug resistance

Potential mechanism of drug resistance include: (i) conversion of the drug to an inactive form by an enzyme; (ii) modification of a drug sensitive site; (iii) increased efflux or decreased influx; (iv) alternative pathway to bypass inhibited reaction; (v) increased production of drug sensitive enzymes; (vi)
increase in the amount of an enzyme substrate (i.e., to compete with the drug); (vii) decrease requirement for product of inhibited reaction; and (viii) failure to activate the drug.

Many of these mechanisms have been observed in parasites such as alteration in cell permeability, modifications of drug sensitive sites and increased quantities of the target enzyme. These modifications may arise in a population of parasites by a number of mechanisms such as (i) physiological adaptations; (ii) differential selection of resistant individuals from a mixed population of susceptible and resistant individuals; (iii) spontaneous mutations followed by selection; and (iv) changes in gene expression (gene amplification).

It is believed that the genes which confer resistance to a particular drug are already within the parasite populations, but prior to exposure to the drug, are present at low frequencies. Exposure to the drug selects for these resistant individuals and their frequency within the population increases rapidly. The appearance of resistance within a population has been observed to occur within 5-50 generations. One factor which might cause the rapid acquisition of resistance is the sub-optimal concentration of the drug, resulting in more survivors.

Drug resistance in Leishmania

Resistance of Leishmania against a given drug may be either natural, or may be acquired when the parasites are exposed to sub-optimal drug doses. Resistant phenotypes selected in vitro are usually obtained by culturing wild type parasites under step-wise increasing drug concentration.

Leishmania has the potential to respond to drug pressure in multiple ways. Most of the understanding in this area is gained from work based on cells in which resistance was selected in vitro. Toxic metalloids such as arsenic and antimony have always been an integral part of natural environment. Metalloid containing drugs are used to combat infectious diseases caused by pathogenic parasites, as well as in anticancer therapy. To survive in such a hostile habitat, it is crucial to develop strategies to exclude toxic substances from the cell and to acquire tolerance. Cells remove metalloids from the cytosol either by active efflux or by sequestration in an integral organelle. Controlling the influx appears to be another way of maintaining a low intracellular metalloid content. The presence of metalloids could also activate transcription of various cellular defence genes. The emergence of resistance to metalloid containing drugs is a serious threat to effective medical treatment. It is therefore, important to identify the components that cause the resistance phenomenon.

Leishmania cells have been selected in the past for Sb(V) resistance, and some resistance mechanisms were suggested, including reduced accumulation, gene amplification, and loss of reduction of the metal. Since the active drug is likely to be Sb(III), cells were also selected for Sb(III) resistance, and analysis of these mutants led to the proposal of a model for resistance. This model was derived mostly from work carried out while studying resistance mechanisms to arsenite, a metal sharing several characteristics with antimony, but seems to hold true for Sb(III), at least in L. tarentolae promastigotes. Once Sb(III) is within the cell, it would be conjugated to trypanothione, the parasite specific spermidine glutathione conjugate. Trypanothione is found to be increased in arsenite and antimoniate resistant cells. This Sb-trypanothione conjugate could then be sequestered inside a vacuole by the ABC transporter PGPA or extruded from the cell by a thiol-X pump. Altered transport of metals appears to be an important determinant for resistance. An altered membrane partition model for decreased drug accumulation in drug resistant cells has been described. This model suggests that decreased drug accumulation is the result of alterations in pH gradients, electrical membrane potential and perhaps other biophysical parameters, and is not necessarily a direct result of drug trafficking. Drug resistance is also associated with changes in physiological events such as parasite infectivity, incorporation of metabolites, xenobiotics conjugation and traffic, intracellular metabolism, host-parasite interaction, parasite cell shape and promastigote-amastigote differentiation. An understanding of these
physiological events may be helpful for designing chemotherapeutic approaches to multiple cellular targets, identifying strategies to circumvent Leishmania drug resistance. Transport studies of Leishmania cells selected for SbIII resistance in vitro (either as promastigotes or amastigotes) have measured a reduction in accumulation of metals in resistant Leishmania. Either reduced uptake or increased efflux could explain the reduced accumulation\(^{25,34}\). It has been shown that a metal efflux pump is present in the Leishmania plasma membrane\(^{31}\) which recognizes the metal conjugated to thiols, such as glutathione or trypanothione\(^{29,31}\). In mammalian cells, thiol associated efflux is mediated by the ATP binding cassette (ABC) transporters of the multidrug resistance protein (MRP) family\(^{35,36}\). These are involved in multidrug resistance as well as metal resistance\(^{37,38}\). Three different classes of ABC transporters have been described in Leishmania parasites. The first group is homologous to the human multidrug resistance protein (MRP) subfamily of transporters\(^ {39}\). The second class of ABC proteins identified in Leishmania parasites is transporters with a higher similarity to mammalian P-glycoproteins that confer a multidrug resistance (MDR) phenotype similar to that observed in cancer cells\(^{40,41}\). The third class of ABC transporters has been reported to show high homology with members of the mammalian ABCA subfamily with related gene been identified in L. tropica\(^ {42}\).

**Drug resistance in clinical isolates of L. donovani**

So far ours has been the only attempt to elucidate the molecular mechanism of resistance to antimony in field isolates. A lot of knowledge has been generated on work with laboratory mutants but the most pertinent question is whether this knowledge can be translated to the field. A better understanding of drug resistance mechanisms in the field will allow the development of new diagnostic assays, such as nucleic acid based tests, that could rapidly detect a resistance gene.

The first task is to confirm whether the field isolates are truly resistant to antimonial therapy or not\(^ {43}\). The field isolates from drug unresponsive and responsive VL patients were collected by us from disease endemic areas of Muzaffarpur in Bihar and Varanasi between 1995-1998; 1999-2000; 2003-2004 and 2005. The patients were admitted to the Kala-azar Medical Research Centre of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh and also its affiliated hospital situated at Muzaffarpur, Bihar. The criterion for diagnosis was the presence of Leishman Donovan (LD) bodies in splenic aspirations performed and graded according to standard criteria\(^ {44}\). After diagnosis, the patients were administered a course of sodium antimony gluconate, 20 mg/kg body weight intravenously once daily for 30 days. Response to treatment was evaluated by a repeat splenic aspiration on day 30 of treatment. Patients were designated responsive based on the absence of fever, clinical improvement with reduction in spleen size and absence of parasites in the splenic aspirate while patients who showed presence of parasites in splenic aspirates were labelled as antimonial unresponsive. These patients were subsequently treated successfully with amphotericin B.

Splenic aspirates of responsive (S) and unresponsive (R) kala-azar patients (VL) were adapted in vitro culture as described\(^ {43}\) and cultures maintained at 26°C. In order to determine whether resistance is the intrinsic property of parasites and not the host, antimonial drug sensitivity of these isolates were evaluated in vitro\(^ {43}\). A correlation between clinical response and SAG sensitivity in vitro was observed (Fig. 1). Isolate R1 was more resistant than isolate R2 and R3, and isolate S in comparison was drug responsive.

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**Fig. 1.** Sodium antimony gluconate (SAG) sensitivity of Indian Leishmania donovani isolates (S) sensitive; (R1-R3) resistant, assayed as amastigotes in J774 macrophages.
Fig. 2 (A). A confocal image. Expression of green fluorescent protein (GFP) in electroporated *Leishmania donovani* field isolate. Cells were viewed after washing and fixing in 3.8 per cent formaldehyde in phosphate buffer saline (PBS).

Fig. 2 (B). Confocal image of intracellular amastigotes expressing GFP from episomal vector.
Since classic techniques for detecting the susceptibility of Leishmania to different drugs are time-consuming, laborious and require the use of macrophages, we introduced the green fluorescent protein (GFP) into the field strains of Leishmania. Leishmania expression vector pXG-GFP was obtained as a gift from Dr Stephen Beverley (Washington University, St. Louis, MO).

The transfectants were examined for green fluorescent protein (GFP) expression by confocal microscopy. As shown (Fig. 2A) GFP was completely localized in the cytosol. Cells were viewed after washing and fixing in 3.8 per cent formaldehyde in phosphate buffer saline (PBS). These promastigotes were used to infect J774 macrophages at a host-parasite ratio of 1:10. Again using confocal microscopy, the intracellular amastigotes expressing GFP from episomal vector could be visualized directly without the need for any cumbersome antibody staining step (Fig. 2B).

Flow cytometry (FC) was used to quantify the parasitocidal effect of commonly used antileishmanial drugs. Thus, using GFP transfected clinical isolate of Leishmania donovani, we demonstrated the parasitocidal effect of drugs as reported in literature but in a easier, reliable, simple, less expensive and quick way. This rapid determination of drug activity as done by FACS required no additional antibody staining. This in vitro model presents numerous advantages over the traditional drug screening procedure or over the luciferase system as it requires neither substrates nor cofactors due to the intrinsically fluorescent nature of the protein. Although animal models are well established for drug testing, they are not suitable for large-scale primary drug screens. Since cells are not killed during this procedure, which makes possible high throughput screening (HTS) in microtitre plates. Currently it is being automated in 96-well formats for high-throughput screening of compounds from libraries in our institute.

**Fig. 3.** Plasmid constructs. Leishmania-specific vector P6.5MCS was used for cloning the amplicon in 2 different orientations with reference to that of nagt (amplicon in correct orientation with respect to nagt of Leishmania expression vector (C) and amplicon in reverse orientation with respect to nagt of Leishmania expression vector P6.5 (P6.5 R)).
Having established parasite resistance to the antimonial drug (SAG), these isolates form the basis of our further work to characterize the molecular and functional basis of resistance.

Nuclear DNA was isolated from these parasites, using DNAzol (Gibco-BRL, Gaithersburg, MD) according to the manufacturer’s instructions, and then digested with HindIII or EcoRI, which recognize AT-rich sequences (and therefore cause relatively few breaks in Leishmania DNA, with its 65% GC content). When the HindIII fragments were subjected to electrophoresis in agarose and stained with ethidium bromide, leishmanial DNA from the unresponsive cases was found to contain a 1.254 kb fragment which was not present in the lane corresponding to the responsive isolate. It then seemed possible that this fragment was linked to the drug resistance in the unresponsive isolate. In order to establish a link between SAG resistance and the 1.254 kb sequence, transfection studies were carried out\(^7\). The SAG resistance of the transfectants in which the test sequence had been correctly oriented with respect to the \textit{nagt} gene was compared with that of transfectants (used as controls) which carried the sequence in the reverse orientation (Fig. 3). Resistance was explored both while the parasites were amastigotes in J774 macrophages and after such amastigotes had been allowed to differentiate into promastigotes. In six independent experiments, the transfectants with the correctly oriented sequence were consistently found to be 2-3 fold more resistant to SAG than the control \textit{L. amazonensis} (Fig. 4).

**Molecular mechanism(s) responsible for resistance in clinical isolates of \textit{L. donovani}\(^7\)**

The amplified sequence detected in antimonial unresponsive patients did not hybridize with probes derived from ABC transporter genes which have been implicated to be involved in antimony resistance in laboratory mutants. Nor did the amplified sequence (AF 273843) show any homology with the sequences for proteins already associated with drug resistance in \textit{Leishmania} laboratory mutants. Thus it can be affirmed that the mechanisms of resistance postulated for laboratory mutants of \textit{Leishmania} were probably not operating in the present field isolates of \textit{L. donovani} from India\(^7\).

![Figure 4](image_url)

**Fig. 4.** Increased resistance to sodium antimony gluconate (SAG) in \textit{Leishmania amazonensis} amastigotes as a result of transfection with the 1.254-kb sequence. Macrophages of the J774 line were infected with promastigotes of transfectants in which the gene sequence was placed in the correct or reverse orientation with respect to the \textit{nagt} gene of the \textit{Leishmania} expression vector P6.5. After 3 days in tissue-culture flasks, the infected cells were plated at 0.5 million cells/well, treated with graded concentrations of SAG, and then incubated for another 24 h before the numbers of amastigotes were estimated. The total number of amastigotes/well was estimated from the number of macrophages, the percentage of macrophages infected and the mean number of amastigotes/infected macrophage. Vertical lines indicate SD.

An open reading frame of 305 nucleotides was identified within this novel sequence. The entire sequence consisted of mostly hydrophobic amino acids. Sequence analysis did not convincingly support the presence of putative transmembrane domains that are essential features of transport proteins. Efflux therefore seems unlikely. No obvious BLAST hits with any protein implicated in drug resistance emerged with this sequence. This corroborated the fact that we had not obtained any signals on hybridization of this sequence with probes implicated in drug resistance in laboratory mutants. Therefore, this gene appears to be conferring resistance by a novel mechanism. One structural feature that may suggest how this sequence confers resistance was the prediction of phosphorylation sites.
Ser 0, Thr 3 and Tyr 1. It has been reported that P-glycoproteins and homologous multidrug transporters contain a phosphorylated linker sequence that is proposed to control drug efflux (Fig. 5). Phosphorylation is enhanced in resistant strains compared with wild type strains. The predicted phosphorylation sites in our sequence awaits experimental demonstration. It may be that protein phosphorylation is playing a role in signal transduction pathway in the parasite after exposure to drug and thus confers resistance. We have done the cloning, expression, purification and antibody production of this gene implicated in imparting resistance. The expressed recombinant protein was found predominantly in the insoluble fraction. This could be caused by strong membrane association of the recombinant protein since it is highly hydrophobic. Antibody raised against this recombinant protein was localized on the cell surface in the resistant parasite (Fig. 6). Now that the \textit{Leishmania} genome (\textit{major} and \textit{infantum}) has been sequenced, we carried out a search to identify whether we could detect any homologous gene to that of the gene we have found in the clinical isolate which seems to be associated with the resistance phenotype. We found the gene with 80 per cent homology on chromosome 18 in both the species (Fig. 7). The protein is hypothetical but conserved. Our work so far suggests that this gene is responsible for resistance induced modification of membraneous lipid in the parasite cell.

Functional studies to elucidate mechanism(s) responsible for resistance in clinical isolates of \textit{L. donovani}

It has been discussed above that one of the pathways adopted by the parasite to develop resistance to antimonials has been attributed to over-expression of ABC efflux pumps (Fig. 8). Although, it is conceivable that other mechanisms, unrelated to drug efflux, might be involved in \textit{Leishmania} resistance to antimonials. To date, an unanswered yet pertinent question is the status of the MDR or MRP ABC transporter pumps in clinical isolates collected from VL patients. Using flow cytometry, we have assayed the functional activity of MRP and MDR in clinical antimony resistant and sensitive \textit{Leishmania} isolates (unpublished observation). Our studies indicated that the clinical isolates tested were all conspicuous in the absence of the classical MDR pump which is inhibited by verapamil. An
increased MRP like pump activity was however detected in the resistant clinical isolates although resistance was not solely reflective of this as the isolate from which the gene seemingly involved in resistance has been cloned and expressed, showed no MDR or MRP pump activity. We have already seen that resistance induced modification in the parasite cell surface could be a contributor towards this phenotype. We also found increased thiol levels in the resistant isolates that possibly generates increased formation of metal thiol adducts (unpublished). Taken together, our results suggest that the clinical isolates use various type of mechanisms simultaneously.

**Microarray analysis of differential gene expression in antimony resistant kala-azar clinical isolates**

DNA microarrays are powerful tools employed in the field of parasitology. Studies have validated
the use of microarrays in studying drug resistance in *Leishmania* and have pinpointed new genes overexpressed either by gene amplification or by other mechanisms. We made use of DNA microarrays as a rapid screening tool to identify genes associated with phenotype of interest, i.e., drug resistance. Once target genes are identified, additional laboratory resources may be invested to validate the list and to further characterize the relationship of their biological functions to the process of drug resistance. We obtained microarrays of cDNAs from both the stages in the life cycle of the parasite promastigote and amastigote. To probe these microarray slides, fluorescent cDNAs were synthesized from total promastigote RNA isolated from drug unresponsive and responsive clinical isolates of *L. donovani*. The Cy5 signal (from drug unresponsive isolate) was plotted against the Cy3 signal (from drug responsive isolate) on a scatter plot (Fig. 9). For each gene represented in the array, the Cy5/Cy3 fluorescence ratio measured at the corresponding array element is a quantitative measurement of the relative abundance of the transcript of that gene in the two isolates.

The distribution was not tightly packed with a fraction of the points deviating from line of best fit indicating possible differential expression. The significantly expressed genes in the resistant isolate were further compared bioinformatically and clustering of cDNAs with a common pattern of expression revealed potential pathway specific genes. Though majority of genes were detected as hypothetical proteins, the analysis by microarray has shown a set of genes whose expression was significantly altered compared to sensitive (unpublished). These identified genes belonged to the ABC transporter family and also genes pertaining to cell surface molecules again indicating to resistance induced modification therein.

**Fig. 9.** Gene expression analysis of resistant isolate as determined by DNA microarray. (A) Scatter plot of hybridization intensities between resistant Cy5 and sensitive Cy3 isolates. The expression of genes represented within the dashed lines are considered as similar in the two isolates. Dashed lines indicate two fold differences and genes whose expression differ significantly are indicated.
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