Studies of Gene Expression in Drug Resistant *Leishmania donovani* using DNA Microarrays

THESIS

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By

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Under the Supervision of

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CERTIFICATE

This is to certify that the thesis entitled '**Studies of Gene Expression in Drug Resistant *Leishmania donovani* using DNA Microarrays'** and submitted by Dhiraj Kumar ID No. 2004PHXF426 for award of Ph.D. Degree of the institute embodies original work done by him under my supervision.

Signature in full of the Supervisor _____

Name in capital block letters Dr. POONAM SALOTRA

Designation Deputy Director

Date 17/02/2010

Acknowledgement

Dealing with disease is the biggest challenge of Life in my experience. The blow of severe illness makes time stand still, priorities change, and Life takes on new meanings. The patient feels helpless, is overcome by feelings of insecurity and fear for losing his grip on Life; his loved ones feel frustrated and powerless. It lies in our human nature to fight back disease, trying to regain control over our lives. If you are Lucky there is a medical treatment to help you in that fight. Treatment gives hope; if treatment fails, that hope is shattered again.

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Introduction:

Visceral leishmaniasis (VL) or Kala-azar (KA), fatal if not treated, is caused by the *L.donovani* complex comprising of *L.donovani, L.infantum* and *L.chagasi*. More than 90% of the VL cases in the world are reported from Bangladesh, Brazil, India and Sudan (Desjeux, 2001). In India, states of Bihar, Uttar Pradesh and West Bengal are highly endemic foci of VL where periodic epidemics are common (Adhya et al., 2002). Over 60% of the cases in Northern Bihar are resistant to the traditional antimony therapy and respond only to Amphotericin B (AmB) treatment (Sundar et al., 2000). This is in contrast to other areas where pentavalent antimonials still provide a cure rate of about 95% (Bryceson, 2001). The molecular basis for drug resistance is still not known and further studies are warranted in this area. The prevalence of drug resistance in such high proportions is unique to India. Thus, there is an urgent need to identify targets to study and combat drug resistance in VL, particularly in India.

Drug resistance is a complex phenomenon in the *Leishmania* parasite as several metabolic pathways and membrane transporters are implicated in the resistance phenotype. A targeted DNA microarray with 44 known genes shown to be responsible for resistance has been used to show the linkage of the genes to drug resistance in *Leishmania* parasite (Guimond et al., 2003). Microarray technology has been used successfully to identify critical genes expressed during development of malaria parasites (Hayward et al., 2000) , *Leishmania major* (Saxena et al., 2003), genes differentially expressed in drug resistant and susceptible *Candida albicans* (Rogers et al., 2002) and genes induced by drug in *Mycobacterium* (Wilson et

al.,1999). This methodology is applied here to make a breakthrough in discovering the genetic determinants of drug resistance in *Leishmania*. In this study, we made use of microarray screening as a rapid tool to identify genes in promastigotes of *L.donovani* clinical isolates associated with antimony resistance. The altered expression of these genes were validated by reverse transcriptase-PCR (RT-PCR) and also where-ever possible at protein level. We analysed the expression of selected genes in natural antimony resistant and sensitive isolates of *L. donovani* by Real Time PCR.We identified two genes; either of which upon overexpression in to the sensitive parasites modulated the susceptibility of the parasites not only towards SAG But also to other widely used drugs for VL treatment including AmB and Miltefosine(MLF).

Aims and Objectives

The aim of the work is to define the genetic determinants of drug resistance in Leishmaniasis, which will be pursued by state of the art methods. The microarray technology will be utilized to identify constellations of genes that exhibit altered expression in drug resistant parasite isolates. Our purpose is to identify the genes, which regulate the susceptibility to antimonial compounds. These gene products are most likely to provide understanding of the resistance mechanism of the parasite and would provide information regarding potential biomarkers of resistance or drug targets for VL.

Specific Objectives:

1. *In vitro* assessment of drug sensitivity in clinical isolates of *Leishmania* from Sodium antimony gluconate (SAG) responsive and nonresponsive kala azar patients.
2. Gene expression analysis in drug sensitive and resistant *Leishmania* isolates using genomic microarray and validation of expression by RT-PCR.
3. Sequence analysis for identification of genes associated with drug resistance phenotype.
4. Expression analysis of selected genes in natural antimony resistant and sensitive isolates of *L. donovani* by Real Time PCR.
5. Functional characterization of selected genes to understand their role in drug resistance/susceptibility.

1. Isolation of parasites, determination of drug sensitivity of parasites isolates.

To study drug resistant strains of *Leishmania*, the parasite will be isolated from bone marrow aspirates of drug sensitive and resistant Kala-azar patients, hailing from Bihar and reporting to Safdarjung Hospital, New Delhi. The isolated parasites will be cultured in Medium 199 with 10% fetal calf serum and propagated in the same medium minimally at 26^0C.

The isolates will be characterized as drug resistant or sensitive on the basis of *in vitro* response to the drug. Sensitivity of different parasite isolates to the drug SAG will be evaluated using promastigotes as well as intracellular amastigotes.

2. Gene expression analysis in drug resistant *Leishmania* isolated from Indian kala azar patient using genomic microarray and validation of expression by RT-PCR.

To understand the pattern of gene expression in SAG sensitive and resistant isolates of *L. donovani*, RNA will be isolated from antimony sensitive and resistant *Leishmania* parasites respectively. For transcriptome analysis *L.donovani* genomic microarray chips are available in our lab. The chips have been constructed in collaboration with a lab in CBER, FDA, USA as a part of our Indo US VAP project. A total of 8448 PCR amplified inserts from a library of 1-1.5kb randomly sheared fragments of genomic DNA from a fresh isolate of *L.donovani* prepared from an Indian KA patient ligated into pZeRO vector, representing ~60% of the expressed genes, along with alien external DNA, 24 known *Leishmania* genes and 12 negative controls, were printed in duplicate on poly-lysine slides.

To minimize variations in the RNA quality, the cells will be collected at identical growth points under identical conditions. Fluorescently labeled cDNA will be synthesized from total RNA of drug sensitive and resistant parasites. The two fluorescently labeled cDNAs will be hybridized with the *L. donovani* genomic microarray. The hybridized microarrays will be scanned in a laser scanner (Axon 4000A) and visualize using GenePixPro5.0 software. Data will be analyzed with the help of Acuity3.1 software following LOWESS normalization.

3. **Identification of genes associated with drug resistance phenotype**

Clones with altered expression in drug resistant parasites will be sequenced. The sequences will be searched against Genbank to look for previous identification or homology to known genes.

4. Expression analysis of selected genes in natural antimony resistant and sensitive isolates of *L. donovani* by Real Time PCR.

Expression of selected genes in *L.donovani* field isolates will be analyzed by Real Time PCR. Further, the higher expression will be confirmed at protein level by western with the available antibodies.

5: Functional characterization of selected genes to understand their role in drug resistance/susceptibility.

Selected gene (s) up-regulated in the resistant parasites will be over-expressed in sensitive parasites to investigate if it alters the phenotype from sensitive to resistant.

Review of Literature

General Introduction

The parasite *Leishmania*

History, taxonomy and clinical manifestations

In 1903, Sir Wiliam Leishman wrote of *'..... a species of Trypanosoma as the cause of one of the indefinite varieties of fever occurring in that country (India), in which the presence of malaria parasites in the blood is not determined.....for want of a better name, I may speak of it as Dum-dum fever....'* (Leishman, 1994). Charles Donovan wrote in reply to that report he had seen a similar phenomenon in Indian patients (Donovan,1994). The 'species of *Trypanosoma*' they both had discovered in the spleen aspirates of their patients was called *Leishmania donovani*, and the febrile disease is now known as Dum-Dum fever, kala-Azar (hindi for black fever) or Visceral leishmaniasis (VL).

Meanwhile, more than 20 *Leishmania* species and subspecies have been described that can infect humans (Ashford, 2000).They belong to the family of Trypanosomatidae in the order of Kinetoplastida. The heterogeneity of the *Leishmania* species is reflected in the variety of clinical manifestations they cause in humans, which can be broadly divided in 3 classes (WHO 2000, TDR -WHO).

(i) **Cutaneous Leishmaniasis (CL):** skin lesions on exposed parts of the body; usually self healing but causing extensive scars; the diffuse form results in disseminated and chronic skin lesions and is very difficult to treat.

(ii) **Mucocutaneous leishmaniasis (MCL):** starts with skin ulcers but spreads out and results in dreadful tissue destruction of mouth and nose.

(iii)**Visceral Leishmaniasis (VL):** disseminated visceral infection is fatal if not treated.

All forms of Leishmaniasis are immune-pathologies as the parasite resides intracellularly in macrophages (mφ) and the nature of the host immune response determines the expression and outcome of the disease.

Life cycle of the parasite:

Life cycle

Leishmania are digenetic parasites, in natural conditions they are transmitted by an insect vector (Phlebotomine sand fly) to vertebrate host (mammals, including humans). As they cycle between their two hosts, they change morphology to adapt to the different environments. Two main morphological forms can be distinguished: (i) the slender flagellated promastigotes, which replicates in the gut of the sandfly and (ii) the oval amastigotes, which reside in the reticuloendothelial cells of the vertebrate host.

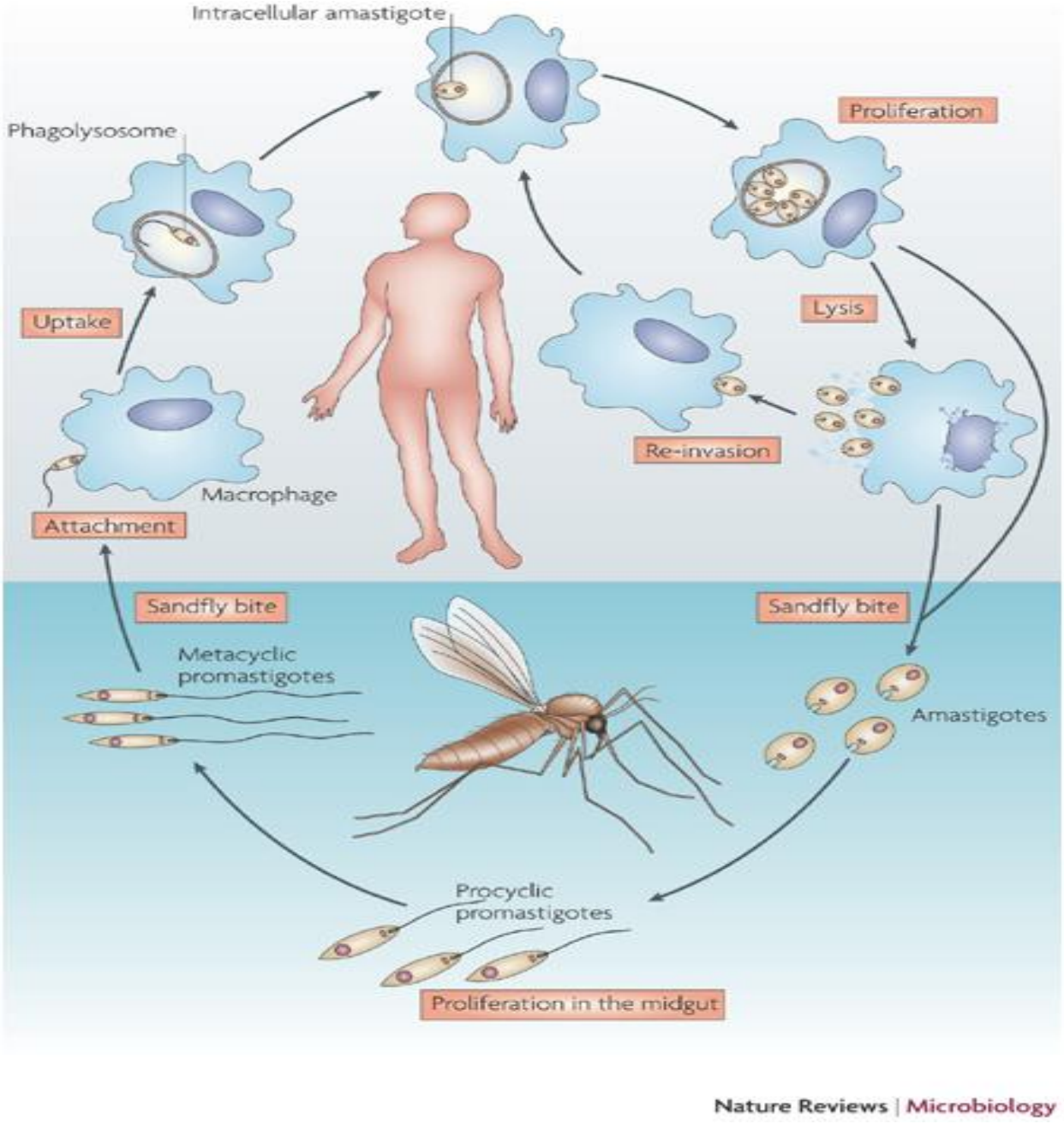


Figure 3.1: Schematic representations of the Life cycle of *Leishmania.*

Female sand flies seek a blood meal at or after dusk, which they require to sustain egg development. When an infected female sandfly takes a blood meal from a mammalian host, she regurgitates infectious (metacyclic) promastigotes at the site of the bite. The promastigotes are

quickly taken up by the tissue phagocytes transform in to the non motile amastigotes and replicate in mφ. When a sandfly feeds on an infected host, the imbibed amastigotes transform in the sandfly gut and replicate as promastigotes, thereby gradually transforming into non-dividing infective promastigotes. The infective promastigotes are then ready to be transmitted to a new mammalian host.

Vector

More than 1000 Phlebotomine sand flies (Diptera, Psychodidae) are known, but only about 70 have been identified as *Leishmania* vectors. Two genera are involved in transmissions to humans: *Phlebotomus* in the Old world and *Lutzomyia* in the New world (Murray et al., 2005). Sand flies are found in a very wide range of habitats, ranging from deserts to tropical rain forests essentially in tropical and sub tropical regions. Phlebotomine sand flies often breed and rest in the same microhabitat, such as the soil accumulated in cracks in walls and rocks, in animal burrows and shelters, household rubbish, or in damp leaf litter in forests. These environments provide organic matter, heat and humidity needed for the development of larvae.

Adult sand flies are not active during the day and seek out cool and relatively humid dark niches which allow survival in very hot and dry climates. They become active at dusk when the ambient temperature drops and humidity rises. The female sand fly then searches for blood meals and thereby covers a radius of a few to several hundred meters around its habitat. The typical micro focal life style of the *Leishmania* vector is reflected in 'Pocket infection' patterns in endemic villages; it is mostly the mammalian host that contributes to the spreading of the parasites.

Reservoir and transmission modes

Parasite populations are maintained in their mammalian hosts, the reservoirs for transmission. The type of host reservoir and its environment determines the transmission mode of the disease to humans. Essentially 3 different transmission modes can be distinguished in leishmaniasis:

(i) **Zoonotic transmission:** Parasites essentially circulates between wild animal host and sand flies in primary environment, humans are accidental hosts, e.g. infections with *L.(V.) braziliensis* in Amazonian forests.

(ii) **Anthropo-zoonotic transmission:** parasite circulates between animals (wild and domestic) and humans. This transmission mode is typically for secondary environments, e.g. infections with *L. (L.) infantum* in Mediterranean region with stray and domestic dogs as reservoir.

(iii) **Anthroponotic Transmission:** parasites essentially circulate between humans in domestic environments, e.g. *L. (L.) donovani* in the Indian subcontinent and East Africa.

Prevalence and geographical distribution:

The leishmaniasis is globally widespread, prevalent in a total of 88 countries, of which 72 are classed as developing countries. There are a total of 350 million people at risk with 12 million affected by leishmaniasis. Of all VL cases, 90% occur in just five countries, Bangladesh, India, Nepal, Sudan and Brazil. As for CL, 90% occurs in seven countries, Afghanistan, Algeria,

Brazil, Peru, Saudi Arabia and Syria. MCL is mainly endemic in Bolivia, Brazil and Peru (WHO 2000, TDR -WHO).

Unusual Biological characteristics of Trypanosomatids

Trypanosomatids are among the most ancient eukaryotes, their linkage of *Kinetoplastida* extends farther back than those of animals, plants and fungi (Fernandes et al., 1993; Beverley, 1996). Their evolutionary origin is reflected in a number of unusual biological characteristics including:

(i) The presence of kinetoplast, a special part in their single mitochondrion where DNA exists of thousands interlocking mini-circles, serving in the complex process of mitochondrial RNA editing (Benne, 1986),

(ii) Compartmentalisation of energy metabolism, with the glycolytic pathway and some other enzyme systems sequestered in separate organelles, the glycosomes (De Souza, 2002),

(iii) A redox- system based on a unique thiol called trypanothione (Fairlamb, 1985),

(iv) Polycistronic transcription and post-transcriptional regulation of gene expression (Johnson, 1987; Myler, 1999.).

Gene organisation and transcription

The *Leishmani*a genome is a relatively small Eukaryotic genome with an estimated size of $3.5X10^7$ bp and is organized in 34-36 chromosomes, ranging in size from 0.3 to 2.8 Mb. Old World *Leishmania* (*L. donovani* and *L. major* groups) have 36 chromosome pairs (0.28 to 2.8 Mb) (Wincker et al., 1996), whereas New World species have 34 or 35, with chromosomes 8+29 and 20+36 fused in the *L. mexicana* group and 20+34 in the *L. braziliensis* group (Britto et al.,

1998). The chromosomes contain repetitive telomeric sequences but do not condense during mitotic cycle.

Leishmania is a diploid organism and functionally asexual. *Leishmania* multiply by binary fission, an assumption of sexual reproduction naturally arises from the existence of diploid genome. The existence of self-fertilization mode of replication has been suggested to account for the contrast between the high numbers of variants in the chromosome 1. In addition natural *Leishmania* hybrids have been described in isolates from Old and New Worlds. Despite this evidence, sexual exchange has not been shown experimentally in *Leishmania* and the available data indicate that genetic exchange in the wild might be infrequent at best. Population genetic studies indicate that the two gene consequences of sexual reproduction, segregation and recombination are absent in *Leishmania*, and a clonal, asexual model has been proposed for *Leishmania* populations.

Genome structure and content

The 32,816,678–bp genome sequence of *L. major* released in July, 2005 were obtained by shotgun sequencing large-insert clones and purified chromosomal DNA (Ivens et al., 2005). A single contiguous sequence was generated for each of the 36 chromosomes although the "right" end of chromosome 8 lacks a small amount of sub-telomeric sequence and telomeric hexamer repeats. Although the genome is partially aneuploid (Sunkin et al., 2000) and there are three large scale allelic differences, there are very few (<0.1%) sequence polymorphisms. Analysis of the *L. major* sequence using several algorithms predicts 896 RNA genes, 71 pseudogenes, and 8370 protein coding genes, of which 3083 cluster into 662 putative families of related genes. Most of the smaller (<10 members) gene families appear to have arisen from tandem gene

duplication, whereas most members of larger (>10 members) families have multiple loci containing single genes and/or tandem arrays; many of the latter contain *Leishmania*-specific genes. Out of ~8379 genes, 310 are experimentally characterized, the identity of 2610 genes is inferred from homology to other databases, 4674 genes code for conserved hypothetical proteins, 690 sequences are orphan meaning no predicted function can be attributed to them and 71 genes are termed as pseudogenes and have premature stop codons and/or frame shifts. The *L. infantum* genome contains 8184 genes present on 36 chromosomes (last update March 2008) and *L. braziliensis* 8312 genes present on 35 chromosomes (last update Jan 2007). Full annotations of the genes of both species are under processing. The difference in chromosome number is due to the fusion of chromosomes 20 and 34 to make a single chromosome in *Leishmania braziliensis*.

Chromatin re-modelling

L.major and other Trypanosomatids contain multiple copies of the four core histone genes (H2A, H2B, H3, and H4) and linker histone genes (H1), which package chromosomal DNA into nucleosomes in eukaryotes and regulate access by the RNA polymerase transcription complexes. The H2A variant is a homolog of the highly conserved H2A.Z, which protects "active" chromatin from silencing in yeast. The H2B, H3, and H4 variants appear to be novel, but may have roles in gene silencing, gene expression, DNA repair, and centromere function. The genomes encode a number of enzymes viz. two families of acetyltransferases, at least three families of methyltransferases, and all three known classes of histone deacetylases, involved in histone modification that may influence transcription, replication, repair, and recombination. The parasites possess a range of chromatin remodeling activities typical of eukaryotes, although there are some notable differences (Ivens et al., 2005).

Transcription

Little is known about the mechanism of transcription initiation in *Trypanosomatids*, and only a few promoters have been functionally analyzed (Clayton, 2002). The chromosomes are characterized by their unique arrangement of directional gene clusters (DGCs) in *L. major* (Myler et al., 1999; Worthey et al., 2003). The *L. major* genome is organized into 133 clusters of tens to hundreds of protein-coding genes, with unrelated predicted functions, on the same DNA strand. The clusters can span up to 1259 kb and are separated by 0.9- to 14-kb divergent or convergent strand-switch regions, which show an unusual base composition (Tosato et al., 2001). Experimental evidence suggests that polycistronic transcription by RNA polymerase II (RNAP II) initiates bi directionally within the divergent strand switch regions (Worthey et al., 2003) and terminates within the convergent strand-switch regions, which often contain tRNA, rRNA, and/or snRNA genes. Several long DGCs contain intervening tRNA or snRNA genes (which are transcribed by RNAP III), suggesting that they may represent more than one polycistron. At 55 of 72 chromosome ends, transcription proceeds toward the telomere, and in 12 cases, the DGC closest to the telomere is very short (one to three genes). The RNAP I, II, and III components differ appreciably from those in other eukaryotes. Few potential homologs of RNAP II basal transcription factors found in other eukaryotes could be identified; the three subunits of TFIIH present also function in DNA repair and cell cycle control. TRF4 (which is related to TATA-box binding protein component of TFIID and TFIIIB) is essential for transcription by all three RNAPs. *L.major* genome contains a disproportionately high number of proteins with CCCH-type zinc finger domains, which are found in RNA-binding proteins. These findings, along with the polycistronic gene organization and paucity of RNAP II initiation sites, are consistent with

posttranscriptional control mechanisms being the primary determinants of gene expression (Clayton, 2002).

RNA processing and Gene expression regulation

Trypanosomatid mRNA processing is distinctive: in addition to the trans-splicing of a spliced-leader RNA to the 5' end of almost all mRNAs, the site of polyadenylation is determined by trans-splicing of the downstream mRNA, rather than by an AAUAAA and downstream G/U-rich tract. Only four cases of cis-splicing could be identified. Both cis- and trans splicing appear to be catalyzed by the spliceosome. All snRNAs and most spliceosomal proteins have been identified, along with many putative splicing regulatory proteins, including those containing domains for 3' splice site and branch point recognition factors, as well as several heterogeneous nuclear RNP (hnRNP) and sarcoplasmic reticulum (SR) proteins implicated in splicing and alternative splicing (Ivens et al., 2005). The absence of an RNAPII C-terminal domain may reflect the polycistronic transcription and the resultant uncoupling of transcription termination and polyadenylation.

The exact mechanisms of regulation of message abundance in *Leishmania* are not clear, several salient points have emerged. Regulation of transcript abundance appears to be post transcriptional and is dependent on 3' UTR and intergenic sequences. It is not clear if intergenic sequences functions via their effects on mRNA processing or by binding of stability / degradation factors. In the absence of characteristic eukaryotic modulation of primary transcription via individual promoter activity, post transcriptional mechanisms are the major determinants in the regulation of mRNA abundance in *Leishmania*. The change in steady state levels of regulated transcripts during *Leishmania* development is not accompanied by increased

transcription initiation. The rate of nascent transcript production by isolated nuclei in nuclear run on assay is unchanged in different developmental forms, in spite of dramatic differences in mRNA abundance for the *L.donovani* A2 antigen and spliced leader RNA, *L. chagasi* gp63 and gp46 and *L. donovani* and *L. mexicana* glucose tranporters, as well as *L. infantum* HSP70 and *L. amazonensis* HSP83 genes under heat shock conditions (Aly et al., 1994; Ramamoorthy et al., 1995; Charest et al., 1996; Beetham et al., 1997). Post transcriptional regulation of steady state transcript levels ultimately involves a process of message stability degradation and, by inference, the existence of protein factors and their recognition sequences within the transcripts. The interplay between these factors and their binding sites during the *Leishmania* life cycle could be regulated at the level of trans-splicing and poly adenylation of primary transcripts in to mRNA, or by differential expression of the protein factors affecting the degradation/ stability of precursor or mature transcripts.

Degradation of mRNAs is important in regulating trypanosomatid gene expression, and appears to resemble the situation in mammals, in which the exosome plays a dominant role. Homologs of the deadenylation complexes, in addition to two poly(A) binding proteins and a helicase and the exonucleases required to degrade decapped mRNAs, are present in *Leishmania* genome. The paucity of genes for transcriptional regulation implies a reliance on posttranscriptional control of gene expression (Clayton, 2002) and is consistent with the presence of numerous genes (~40) for proteins with RNA-binding motifs.

Translation and co-/post translational modification

Major components of the translation machinery are found in the *Leishmania* with similar copy numbers (one to seven) as observed in other lower eukaryotes. However, there appears to

be paralogous expansion of the eIF-4A gene, with 15 copies showing 30 to 57% amino acid identity to that from *S. cerevisiae*, and ~100 with <30% identity. Mostly contain adenosine 5'-triphosphate (ATP)–dependent DEAD-box RNA helicase domains, implying nucleic acid binding, perhaps for transcriptional or translational processes. There are also numerous copies of eEF-1a, which complexes with guanosine 5'- triphosphate (GTP) and aminoacyl-tRNAs for ribosomal A site binding during translation, but also functions in processes such as actin binding/bundling in cytokinesis in *Tetrahymena* (Numata et al., 2000). Functionally, *L. major* eIF-2B is predicted to also have mannose-1-phosphate guanyltransferase activity (Garami and Ilg, 2001), whereas the eEF-1B complex has trypanothione S-transferase and peroxidase activity (Vickers et al., 2004). Thus, the expanded number of potential translation factors suggests a high degree of specialization. Protein modification within *Leishmania* involves typical eukaryotic processes, including phosphorylation, glycosylation, and lipidation for stabilization and/or activation. Several major modifications have been well characterized and shown to be essential, namely, glycosylphosphatidylinositol (GPI)–anchor addition, acylation (including N-myristoylation and palmitoylation), and prenylation, all of which facilitate membrane attachment and/or protein-protein interactions. The presence of substantial number of proteins containing motifs for putative N-myristoylation or prenylation, suggests that the enzymes that catalyze these modifications may be promising drug targets, given their large number of possible substrates.

Interaction *Leishmania*-host macrophage

Inoculation of host & uptake by macrophages

Extracellular life in host

Immediately after inoculation, but before entry into their host cells, promastigotes are exposed for a short while to the serum. This is a toxic environment for an extracellular pathogen due to the presence of the lytic factors of the innate immune response. Probably most of the parasites are rapidly killed by complement factors, but a sufficient number survives as they are relatively resistant to complement mediated lysis. (Sacks, 1984; Roberts, 2006). Various surface molecules of *L. (L.) donovani* promastigotes interact with complement factors (binding, cleavage or phosphorylation) thus preventing lytic attack of the complement C5b-C9 complex (Puentes,1989; Hermoso,1991; Brittingham,1995; Bogdan,1998). The vector also assists in the survival of metacyclic promastigotes in the blood stream. The sandfly saliva contains a peptide called maxadilan which inhibits production of pro-inflammatory cytokines and nitric oxide by macrophages (Hall, 1995; Bozza, 1998).

Phagocytosis

While subverting harmful effects of the complement system, *Leishmania* at the same time needs the complement factors for opsonisation and internalisation. *In vivo* opsonisation by C3b and C3bi leads to ligation with the macrophage receptors CR1 and CR3 respectively. Since the *Leishmania* metalloprotease gp63 efficiently converts C3b to C3bi (Brittingham,1995), it appears that CR3 is the predominant receptor, while the interaction with CR1 is only transient (Kane, 2000). There are numerous other receptors which can participate in the uptake of the

parasites including the mannose-fucose receptor binding *Leishmania* LPG , the fibronectin receptor binding gp63 (Rizvi, 1988; Brittingham, 1999), CR4 binding LPG (Talamás-Rohana,1990), and the C-reactive protein binding receptor attaching to LPG. It has been demonstrated that the complement receptors CR3 and CR1 are the primary mediators of parasite adhesion (Brittingham, 1999). The availability of multiple receptor system could ensure easy access for the parasite into macrophages, thereby protecting the parasite from getting eliminated while being exposed in the bloodstream.

Upon phagocytosis, it is generally accepted that the parasites reach their intracellular location, the parasitophorous vacuole.

Parasitophorous vacuole

The phagosome which forms around the ingested parasite during phagocytosis is subsequently thoroughly remodelled via fusion with lysosomes and endosomes. The resulting phagolysosomal like compartment is called parasitophorous vacuole (PV), an acidic compartment where the intracellular form of *Leishmania* resides and multiplies (Antoine et al., 1998). During the maturation of this acidic compartment, promastigotes also transform in to the non motile amastigotes. The transformation is associated with:

(i) Morphological changes, the parasite takes on an oval shape with a short flagellum;

(ii) Metabolic changes, to adjust the acidic conditions of the PV;

(iii) Surface composition changes, the expression of the ‘invasion’ molecules LPG and gp63 drops significantly; a new protective coat is expressed which consists mainly of low molecular weight GIPLs (McConville et al., 1991).

Depending on the species, this transformation can take 2-5 days. Some species, including *L. (L.) donovani*, slow down the PV maturation; which is believed to grant the parasite the necessary time to undergo the complex transformation to the intracellular form fully adapted to reside in the acidic PV (Desjardins et al., 1997).

The parasitophorous vacuole is late endosomal in nature, meaning it is an acidic (pH of 4.7-5.2) and hydrolytic environment (Antoine et al., 1990, 1998). Amastigotes are adapted to this hostile milieu and benefit from the nutrient supply present in such a digestive compartment. Other endosomes/phagosomes containing endogenous macromolecules, or phagocytised cellular debris, can fuse with the *Leishmania* PV via the multiple vacuole trafficking pathways. The PV is equipped with the necessary hydrolases and proteases for degradation of the incoming macromolecules to sugars, amino acids, lipids, phosphate and sulphate, which might then readily be incorporated by amastigotes (Burchmore et al., 2001).

Amastigote replication

Once the infectious process is initiated and amastigotes have formed the parasites starts to divide within the PV. Parasite replication is slow with a generation time of approx. 24 hrs and does not seem to cause abrupt rupture of the macrophages (Chang et al., 2003). Infected organs of lesions often contain many heavily parasitized macrophages which must be grossly functionally impaired but nevertheless still maintain their integrity. It is thought that the degeneration of the host cell is inhibited by the parasite; it has been shown that mΦ pro-apoptotic genes are down-regulated in *L. (L.) donovani* infected macrophages (Buates et al., 2001; Chang et al., 2003).

It is generally believed that infected macrophages eventually do rupture and release amastigotes which are then taken up by neighbouring cells. Other studies suggest that amastigotes loaded PVs can move to the periphery of the infected cell, and release the amastigotes over several hours in an exocytosis-like process (Rittig et al., 2000). The released amastigotes use several of the receptors to gain entry into a new host cell. Since, the abundance of gp63 and LPG has drastically changed in comparison to promastigotes; amastigotes must also rely on different mechanism for uptake. One of those mechanisms involves the Fcγ receptor which binds immune complexes of amastigotes opsonised with host IgG. (Guy et al., 1993; Miles et al., 2005) Alternatively, apoptotic infected macrophages could be phagocytised by other macrophages before the amastigotes are released. This means of amastigotes spreading from one host cell to another avoids the exposure to the lytic factors present in extracellular spaces (Chang et al., 2003).

Cellular functions of macrophage inhibited by *Leishmania*

Macrophages are crucial components of the immune system, as they play an important role from initiation till resolution of infection:

(i) as phagocytes, they contribute to the innate immune response,

(ii) as professional antigen presenting cells, they are crucial to the development of the adapted immune response,

(iii) as effector cells of specific Th1 responses, they efficiently kill intracellular pathogens by cytokine-induced ROI/RNI production.

Invading these central cells of host defence essentially enables *Leishmania* to orchestrate the immune response of the host in favour of its own survival. The impact of the parasite's presence on the functionality of macrophages is reflected in impressive modifications of mΦ gene expression e.g. 30-40% of expressed genes in mΦ is suppressed upon *L. (L.) donovani* infection (Buates et al., 2001; Rodriguez et al., 2004). This complex interaction of *Leishmania* with its host cell lays out the basis for the permissive conditions which allow parasite replication and eventually can result in severe immune-pathology.

The following paragraphs give an overview of some specific macrophage functions that are repressed by *Leishmania*. It is important to emphasise that there are marked differences in macrophage/parasite interaction depending on the infecting *Leishmania* species. This is consistent with the observed differences in pathogenesis, host immune response and clinical profile caused by the different species, markedly visceral versus cutaneous species. The discussion will be limited here to the visceralising species *L. (L.) donovani* and *L. (L.) infantum/chagasi*, while other species will only be mentioned when pertinent for comparison.

Reactive Oxygen Intermediates and Reactive Nitrogen Intermediates production

The main entry mode of promastigotes (via the CR3/CR1 receptors) is a 'silent" phagocytic process with minimal membrane disturbance and usually does not lead to a massive oxidative burst (Brittingham et al., 1996). The mechanism is still unclear, but involves inhibition of protein kinase C (PKC) which normally signals the assembly of the NADPH oxidase complex. LPG also protects against the toxic effects of ROI present in the phagosome as its repeating units are highly effective at scavenging hydroxyl radicals and superoxide (Chan et al., 1989).

Phagosome – endosome fusion

Inhibition of phagosome-endosome fusion is an intra-macrophage survival strategy used by a variety of intracellular pathogens, including *L. (L.) donovani* (Desjardins et al., 1997). As mentioned before, this fusion delay allows the vulnerable promastigotes to transform into amastigotes which are adapted to live in the acidic, hydrolytic PV. The process involves LPG which seems to cause a progressive F-actine accumulation around the phagosomes (Holm et al., 2001). Furthermore, LPG also appears to change the shape of membranes, leading to steric repulsion between the phagosome and the endosome (Olivier et al., 2005).

Cytolysis

The lysosomal enzymes ensure cytolysis and degradation of incoming phagocytised material. Promastigotes can inhibit the hydrolases entering the maturing PV when fusing with lysosomes. The strong negative charge and the repeating units of LPG protect the parasite against a hydrolytic attack. The second invasion-molecule, gp63, is a protease with optimal activity in the acidic PV, and presumably protects the parasite from lysosomal cytolysis and degradation (Seay et al.,1996; Cunningham, 2002;).

Cytokine production

Infection of a macrophage by *Leishmania* influences the cytokine production of that macrophage. Since cytokines are key regulators of the immune system, this cytokine modulating capacity of *Leishmania* contributes to the inhibition of harmful host immune responses. *In vitro* studies have demonstrated that the production of three groups of cytokines is affected upon direct contact/infection with *Leishmania*:

- Pro-inflammatory cytokines:

These cytokines trigger responses resulting in inflammation, a process which promotes the recruitment and adhesion of effector cells and molecules during the host's innate immune response at the site of infection. *L. (L.) donovani* promastigotes inhibit the expression of several pro-inflammatory cytokines in the mΦ, incuding tumor necrosis factor (TNF-α). This suppressed expression probably results from the inhibition imposed by the parasite on the mΦ's signalling pathways.

L.donovani is in general a very poor inducer of inflammation when compared to dermotropic species. For instance, upon *L. (L.) major* infection, there is a more intense recruitment of leukocytes (especially neutrophils) and a concomitant stronger inflammatory response in comparison to *L. (L.) donovani*. The suppressed inflammation of *L. (L.) donovani* infections could favour parasite progression toward its target organs (visceral disease), while *L. (L.) major* would be restricted at the inoculation site (Cutaneous disease) due to the stronger leukocyte recruitment and inflammatory response (Racoosin and Beverley, 1997; Matte et al., 2002).

- T-lymphocyte stimulating cytokines

The cytokine IL-12 is normally secreted by infected macrophages and is an essential cytokine for the development of a specific cellular immune response. Adapted T-lymphocytes also bind IL-12 which leads to secretion of IFN-γ and TNF-α. These two cytokines synergistically activate the mΦ's microbicidal functions through switching on the expression of (i) iNOS and phox (killing the pathogens) and (ii) IL-12 and MHC class II molecules (further development T-lymphocytes).

Numerous studies have demonstrated that macrophage expression of this central cytokine IL-12 is suppressed upon infection with promastigotes and amastigotes of various *Leishmania* species, including *L. (L.) donovani* (Belkaid et al., 1998; Weinheber et al., 1998). The mechanism responsible for IL-12 suppression varies between species and is not completely understood. Presumably, the uptake of amastigotes complexes via Fcγ-receptor contributes to this suppression.

- Anti-inflammatory cytokines:

Leishmania infection also induces production of various anti-inflammatory cytokines, including TGF-β and IL-10, which distorts the normal immune response in favour of parasite survival (Rodrigues Jr et al.,1998; Gantt et al., 2003).

The induced production of IL-10 is a particular critical factor for progression of the disease. This pleiotropic cytokine is the driving force in declining the macrophage's capacity to eliminate the parasite. The effects of IL-10 roughly form the basis of the pathogenesis of *L. (L.) donovani* including:

(i) Inhibition of IFN-γ-induced activation and the concomitant NO-production by mΦ;

(ii) Inhibtion proliferation/development T-lymphocytes during adapted immune response;

(iii) Stimulation B-lymphocyte proliferation and production of IgG antibodies (Belkaid et al., 2001; Murray et al., 2002; Awasthi et al., 2004).

It seems that the produced IgG forms immune complexes with the parasite which bind the Fcγ-receptor (Kane and Mosser, 2000; Kane and Mosser, 2001; Miles et al., 2005) Ligation to this receptor signals parasite clearance to the mΦ. The mΦ subsequently

responds by down regulating IL-12 and up regulating IL-10, in order to prevent further development of inadequate immune responses.

Interaction with T-lymphocytes

T-cell mediated immune responses are crucial to control *Leishmania* infections. Development of this adapted immune response requires reciprocal interaction between *Leishmania* infected macrophage and T-cells. This interaction is also impaired by the parasite on several levels.

Macrophage presenting antigen to naive T-cells:

Phagocytes present antigen/MHC complexes to naive T-lymphocytes in lymphoid organs. The contract between these two cells leads to proliferation/activation of T-lymphocytes if only 2 signals are present; but both signals are impaired in *Leishmania* infected macrophage:

(i) Antigen-MHCII complex (mΦ) ligation to T-cell receptor (TCR): The antigen-MHCII preparation is impaired as amastigotes are protected against cytolytic attack and as such evade the peptide preparation for antigens. *L.donovani* in particular also seems to suppress expression of MHC proteins, both basal and upon IFN-gamma stimulation by T-lymphocytes.

(ii) Co-stimulation by ligation B7.1-2 (mΦ) to CD28 (T-cell): *L.donovani* suppresses expression of co-stimulatory receptor B7.1 and this process seems to involve prostaglandin.

Developed T-cells activating infecting mΦ:

Activated CD4+ve T-lymphocytes exert their effector function through activation of mΦ which leads to up regulated expression of MHC and IL-12 (further T-lymphocyte stimulation) and iNOS/phox (pathogen elimination). Again this activation process requires 2 signals, and again both signals are disturbed by the parasites residing in the mΦ:

(i) IFN-γ secreted by T-cell ligates to mΦ-receptor: IFN-γ responsiveness is paralysed in infected mΦ.

(ii) Co-stimulation by ligation CD40 (mΦ) to CD40L (T-cell); The CD40 signal induces iNOS expression/NO production, but the CD40/CD40L ligation is inhibited. The signalling pathway downstream CD40 is skewed by *Leishmania* towards an anti-inflammatory response, again favouring the survival of the parasite.

Visceral Leishmaniasis

VL is a systemic disease that is fatal if left untreated and is caused by the *Leishmania donovani* complex — *L. donovani* in East Africa and the Indian subcontinent and *Leishmania infantum* in Europe, North Africa and Latin America. The majority (>90%) of cases occur in just six countries: Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil. VL affects poor communities, generally in remote rural areas. The disease is mostly endemic in countries that are among the least developed in the world (such as Nepal) or in the poorest regions of so-called 'middle income' countries (such as Bihar State in India). Patients and families affected by VL become poorer because of the high direct costs (for example, the costs of VL diagnosis and treatment) and indirect costs (for example, loss of household income) of

the disease (Alvar et al., 2006). As India, Nepal and Bangladesh harbour an estimated 67% of the global VL disease burden 20, the commitment of the governments of these countries to launch a regional VL elimination programme is welcome. The target of this programme is to eliminate VL as a public health problem in these countries by 2015, by using a local approach to reduce the annual incidence of VL to less than 1 case per 10,000 individuals.

The clinical presentation of VL

Following an incubation period that generally lasts between 2 and 6 months, VL patients present symptoms and signs of persistent systemic infection (including fever, fatigue, weakness, loss of appetite and weight loss) and parasitic invasion of the blood and reticulo-endothelial system (that is, the general phagocytic system**)**, such as enlarged lymph nodes, spleen and liver. Fever is usually associated with rigor and chills and can be intermittent. Fatigue and weakness are worsened by anaemia, which is caused by the persistent inflammatory state, hypersplenism (the peripheral destruction of erythrocytes in the enlarged spleen) and sometimes by bleeding. The clinical presentation of VL is similar in the various endemic areas but there are some differences. For example, enlarged lymph nodes are rarely found in Indian VL patients but are frequent in Sudanese VL patients (Siddig et al., 1990). Hyper-pigmentation, which probably led to the name kala-azar (black fever in hindi), has only been described in VL patients from the Indian subcontinent, but today this symptom is uncommon and was perhaps a feature of prolonged illness in the era when effective treatment was not available. As the disease advances, splenomegaly can increase, causing abdominal distension and pain, which is sometimes increased by concomitant hepatomegaly. Symptoms and signs of bacterial co-infections such as pneumonia, diarrhoea or tuberculosis can confuse the clinical picture at the time of initial

diagnosis. VL symptoms often persist for several weeks to months before patients either seek medical care or die from bacterial co-infections, massive bleeding or severe anaemia.

VL diagnosis

As the clinical presentation of VL lacks specificity, confirmatory tests are required to decide which patients should be treated. Such tests should be highly sensitive (>95%) as VL is a fatal condition, but also highly specific because the current drugs used to treat VL are toxic. Ideally, a test should be able to make the distinction between acute disease and asymptomatic infection, because none of the drugs currently available is safe enough to treat asymptomatic infections. Moreover, such tests should be simple and affordable.

The golden standard for diagnosis of VL remains microscopic demonstration of *L.(L.) donovani* amastigotes in tissues aspirates (spleen, bone marrow, lymph nodes), the same method Leishman and Donovan used over 100 years ago. The sensitivity varies with the type of tissue used and the technical skill. Spleen tissue has the highest sensitivity (about 98%), but taking spleen aspirates in field conditions is trying and there is a small risk for fatal haemorrhages. Currently, evaluation of treatment relies on this invasive technique in most endemic regions.

Many serological methods have been developed in an effort to replace the invasive parasitological methods. The high antibody titre in Indian VL patients forms a highly sensitive target for diagnostic purposes. Two antibody-detecting tests are now available adapted for use in the field; (i) direct agglutination test (DAT) using freez-dried antigen and (ii) immune-chromatographic dipstick with recombinant leishmanial antigen K39, which is sensitive (90-98%) and specific (93-98%). Especially, the rapid antibody detecting tests are useful in detecting new cases in epidemics. However, such tests cannot differentiate

between active and past infection, and are therefore not useful in evaluating treatment efficacy. Recently we have developed antibody detecting test based on the detection of nuclear phospho- protein Lepp 12 for both prognosis as well as diagnosis of VL. The Lepp 12 antibody titer level remain elevated during active Kala-azar while its level dips down upon treatment, so it has the potential to use as a prognostic as well as diagnostic marker (Kumar et al.,2008).

Sensitive molecular diagnostic methods have been developed, but their applicability is still not feasible in the field. PCR-techniques on blood samples are most useful thought for evaluation of treatment efficacy in HIV co-infected patients in southern Europe. Further efforts are currently being done to simplify the format of PCR-based diagnostic tests by using oligo-chromatography, a dipstick format to detect PCR products could enhance applicability of these molecular tests.

Current treatment options for VL

The term 'treatment options' has only been introduced recently in the field of VL. Until the beginning of the 1990's there was essentially only one treatment widely available, pentavalent antimonials. The loss of antimonials efficacy in North Bihar (India) was a clinical disaster leaving 45% of the world's VL patients in a precarious situation as there was no immediate clear-cut substitute treatment available (Sundar and Murray, 2005). This calamity created awareness in the scientific community on the neglected status of VL treatment and slowly rekindled efforts to generate new treatment alternatives or refinements.

Pentavalent antimonials

Pentavalent antimonials are used to treat all forms of leishmaniasis. The drug is widely accepted and used in most endemic regions as it has passed the test of time (in use since 1940s) and it has a proven efficacy with cure rates > 90%. Pentavalent antimonials can be administered by injection rather than infusion which is a practical advantage. But the intramuscular injections can be extremely painful, because the drug is irritant and sometimes large volumes (e.g. 10 ml for a 50 Kg patient) need to be injected in muscles that are often severely wasted by disease (den Boer et al., 2006). Although administered by injection, patients still preferably need to be hospitalized for 3-4 weeks which sharply increases the cost of treatment. The treatment has a high incidence of adverse effects, particularly in HIV-patients, and long term treatment is restricted by cardiotoxicity(Chulay et al., 1985).

The first published records of use of these trivalent antimonials for treatment were by Macado and Vianna in 1913 for CL and in India in 1915 for VL. The development of the less toxic pentavalent antimonials in the 1920s by Brahmachari, Schmidt, Kikuth and others led to the synthesis of antimony gluconate (Solustibosan) in 1937 and sodium stibogluconate (SSG ; Pentostam) in 1945 (Goodwin,1995). Another carbohydrate complex, meglumine antimoniate (Glucantime), Aventis, soon followed. Solutions of these two pentavalent antimonials, Pentostam (GlaxoSmithKline, UK), containing 100 mg SbV /ml, and Glucantime (Aventis, France) containing 85 mg SbV /ml, remain the first line treatments for VL and some forms of CL (WHO; Steering Committee on Drugs for African Trypanosomiasis, Chagas Disease and Leishmaniasis (ICHEM):Geneva, 1996; Berman,

1997). Generic antimonial products are also available; a recent clinical trial in Sudan has shown the Albert David (Kolkata) Pentostam to be as effective, but 14 times cheaper, than the GSK product (Veeken et al., 2000). Although antimonials remain the mainstay of antileishmanial therapy despite decades of use except in the Indian state of Bihar where resistance is endemic, their mechanism of action, structure and even identity of the biologically active component of the most commonly utilized formulations remain uncertain (Roberts et al., 1993).

As first-line treatment, antimonials have been used largely and were therefore the drug the most exposed to drug resistance risk. Emergence of *L. donovani* resistance to antimonials is now commonplace (Lira et al., 1999). Whereas unresponsiveness to treatment with pentavalent antimonials has long been known, the phenomenon has suddenly increased since the appearance of *Leishmania*/HIV co-infection. In Europe and Brazil, immune-compromised patients infected with *L.infantum* currently show resistance to these drugs, and relapses are frequent. Even in immune-competent hosts primary unresponsiveness to Glucantime® has been observed; the clinical efficacy of pentavalent antimonials seems to decrease during relapses (Faraut-Gambarelli et al., 1997). The most affected area is the region of Bihar State in India where 50–80% cases of VL are resistant to Glucantime® (Sundar et al., 2001). It was therefore necessary to understand the mechanism of resistance to antimonials in order to propose new therapy including reversal agents and other strategies allowing overcoming antimonial resistance. *In vitro* studies have shown that *Leishmania* parasites can develop resistance when cultured in the presence of pentavalent antimonials (Grögl et al., 1989) and parasite cell lines with various degrees of resistance to antimonials

have been developed for both promastigotes and amastigotes *in vitro* cultures (Grögl et al.,1989; Ephros et al., 1997).

Pentamidine

Pentamidine, which is an aromatic diamidine, has been used for many years in the treatment of human leishmaniasis. The isothionate or methanesulphonate salts of pentamidine have been used as second-line drugs in the treatment of visceral leishmaniasis refractory to SbV treatment (Sundar et al., 2002). However, the efficacy of pentamidine or antimony refractory infections has decreased over the years, declining mainly in India, and suggesting that parasites are becoming resistant (Sundar et al., 2000**;** 2001). The toxicity associated with pentamidine including hypoglycemia, diabetes, nephrotoxicity and hypotension has always restricted its use and it is progressively being abandoned as a second-line treatment.However, pentamidine could have a second life since it enhances the activity of other antileishmanial drugs when used in combination. Although pentamidine use has been largely abandoned in India where pentamidine failures are common, it continues to be used alone or in combination with other drugs in other countries (Basselin et al., 1997; Nacher et al., 2001).

Amphotericin B

Amphotericin B (AmB) is a polyene antifungal drug used in the treatment of systemic fungal infections which shows a strong antileishmanial activity. Despite its high efficiency, AmB is also toxic and is associated with severe side effects. However, in areas where there are high levels of SbV resistance, AmB is clearly the drug of choice and resistance to it does not appear to emerge rapidly. Newer lipid formulations of AmB retain their antifungal activity while drastically decreasing toxicity. A parallel situation is seen

when used to treat *Leishmania,* where various lipid formulations of AmB were found to be both highly active and associated with low toxicity (Sundar et al., 2000; 2004). However, lipid formulations of this excellent drug have a prohibitive price tag for developing and emerging countries. Independently of the use of particles for AmB absorption, the challenge in chemistry is now to develop new approaches for AmB use by oral route including for instance 1,3-diglyceride derivatives.

AmB interacts with fungal membrane sterols and preferentially with ergosterol. Like fungi, *Leishmania* also have ergostane-based sterols as their major membrane sterol (Goad et al., 1984) and this likely explains the main efficacy of AmB against *Leishmania*. Several studies confirmed that AmB causes the formation of pores in the membrane that alters the permeability to ions responsible for cell death (Ramos et al., 1994; Azas et al., 2001). In VL, liposomal AmB is more than 95% effective and generally well tolerated; however, its high cost makes it unaffordable in endemic countries (Zilberstein et al., 2002). AmB is mainly used against VL in AIDS patients and post-dermal kala-azar although no cure can be obtained definitely (Freire et al., 1997; Thakur et al., 1997).

To date, no cases of AmB resistance in *Leishmania* infections have been reported in the field although resistant clones of *L. donovani* have been produced in laboratory through stepwise increased concentrations of the drug (Mbongo et al., 1998). A recent report of unresponsiveness to Ambisome in Sudanese patients of VL is worrisome and indicate emergence of AmB resistant parasites (Mueller et al., 2007). In a study with 19 Kala-azar field isolates, we have not observed any clinical resistance but significant increase in the ED_{50} in isolates from high endemicity zone indicated the possibility of emergence of resistance as observed in Sudan (Kumar et al., 2009)

Successive relapses after AmB treatments could contribute to an increase in AmB-resistant *Leishmania* isolates. In the laboratory, the selection of AmB-resistant strains by *in vitro* drug pressure is a long time process suggesting that a long term drug pressure should occur in the field before the mechanisms of resistance take place in parasites. Moreover, the fact that AmB-resistant parasites are poorly infective for rodent presumes that if resistance emerges, resistant parasites would be poorly transmitted in the field. The AmB-resistant parasites have low level of ergosterol, the main target of AmB, possibly explaining the poor affinity of AmB for the modified membranes of AmB-resistant parasites (Mbongo et al., 1998).

Miltefosine

Miltefosine (hexadecylphosphocholine) is an alkylphosphocholine initially developed as an anticancer agent. Miltefosine was first shown to be active against *L. donovani in vitro* (Croft et al., 1987) and rapidly was shown in small studies to have a favourable therapeutic index for Indian visceral leishmaniasis. Miltefosine is therefore the first oral drug proven to be highly effective against VL, including antimony-resistant cases (Jha et al., 1999). However, some drawbacks such as potential teratogenecity and the eventual development of drug resistance (Sundar et al., 2001) require a careful management of its use in the field.

Although few data are available about the mechanism of action of miltefosine, this drug kills the parasite by an apoptotic-like process in *L. donovani* promastigotes (Paris et al., 2004). These results were confirmed in amastigotes (Verma et al., 2004). Miltefosine has been also shown an effect on alkyl-lipid metabolism, and phospholipid biosynthesis (Lux et al., 2000).

As new drug, miltefosine was recently introduced in the field and therefore, clinically resistant parasites have not yet been reported, a significant variation in MIL sensitivity has been demonstrated in clinical isolates of different endemicity region for kala-azar (Kumar et al., 2009). Resistance to miltefosine can be easily selected *in vitro* and some of the resulting mutants have been characterized (Perez-Victoria et al, 2003; Seifert et al., 2003). In miltefosine-resistant *L. donovani* promastigotes, selected for resistance *in vitro,* a reduced accumulation of the drug was observed compared to those in wild-type parasites (Perez-Victoria et al., 2001; 2003a). Such a decreased accumulation was caused by a defect in the inward translocation of the drug, that is, movement from the outer to the inner leaflet of the plasma membrane. One miltefosine transporter, called LdMT, and its β-subunit LdRos3 was recently isolated by functional rescue. LdMT is a novel P type ATPase belonging to the newly described aminophospholipid translocase subfamily (Perez-Victoria et al., 2003). Point mutations at two different positions in the protein were involved in loss of translocation of both miltefosine and glycerophospholipids. Miltefosine is a major breakthrough for the therapy of leishmaniasis; however the ease with which resistant mutants were obtained in the laboratory is worrying. The problem is to know whether mutations in LdMT can also occur in the field. One recommendation in order to slow down the potential emergence of resistant parasites is to use miltefosine in combination with other drugs, such a strategy decreasing drug pressure in the field.

Paromomycin

Paromomycin is an aminoglycoside antibiotic having antileishmanial activity. Paromomycin ointment has proved to be effective for CL treatment but is expensive and of limited availability. This aminoglycoside has also been used in parental formulations for

VL (Thakur et al., 2000). Paromomycin was shown to be effective in a number of small studies and interestingly can be used successfully in combination with antimonials (Thakur et al., 2000).

Paromomycin could have ribosomes as a primary target of *Leishmania* (Maarouf et al., 1995) .It also appears to have other effects including alterations in membrane fluidity and lipid metabolism and may also target key mitochondrial activities (Maarouf et al., 1997).The drug acts on RNA synthesis and modifies membrane polar lipids thereby affecting membrane fluidity and altering membrane permeability (Maarouf et al., 1997). Since paromomycin has not yet been used extensively, resistance is not a problem in the field but resistance to paromomycin was described *in vitro* in *L. donovani* and *L. tropica* strains (Fong et al., 1994; Maarouf et al., 1998).

Sitamaquine

Sitamaquine, a 4-methyl-6-methoxy-8-aminoquinoline (lepidine), previously known as WR6026, is in phase 2b clinical trials for the treatment of VL (Carmen et al., 2008). The drug has broad-spectrum antiprotozoal activity (Yeates 2002.) but with limited clinical use and no reported resistance. In addition, there are no published comparative studies on *Leishmania* species sensitivity.

Control strategies for anthroponotic VL

VL has been identified by WHO as an emerging and uncontrolled disease, this is a reflection of the neglected status of VL in terms of both (i) availability of tools to control the disease and (ii) commitment to implement control strategies. The pillars for control of anthroponotic VL are early diagnosis and treatment, which both act in concert to reduce the

reservoir and thus the transmission rate. But, a combination of poverty-related factors present in the endemic regions prevents both early diagnosis and access to treatment.

Given the anthroponotic nature of the disease, a vaccine could have a major impact on control. However progress in vaccine development is slow and since *Leishmania* has experience in finding ways to tackle the human immune system, vaccine development might prove to be an underestimated challenge.

A second possible strategy is vector control. National programmes of insecticide spraying have been undertaken in the past, but were hampered by expenses and logistics and often not sustainable (Guerin et al., 2002). As the sand flies primarily bite during the night, people can indeed protect themselves while sleeping by using bed nets (Bern et al., 2000). However, sand flies are really tiny (2-3 mm), thus the bed nets need to have a narrow mesh to stop the vectors; such nets are hot and suffocating in tropical climates and thus not accepted. Treatment of bed nets with insecticides repels the sand flies and allows the use of lighter nets. This has been translated in the production of 'long lasting insecticide treated bed nets', in which the insecticide is combined with the material during manufacturing allowing sustainable use. The use of these bed nets is currently under evaluation in the Indian subcontinent (Murray et al., 2005).VL control should be feasibly given the means of diagnosis and treatment are widely available. Especially in a setting like the Indian subcontinent, where VL is largely restricted to defined zones, humans are thought to be the only reservoir and there is only 1 vector (*P.argentipes*). The arrival of the new rapid diagnostic tools (K39-dipstick) and availability of oral drugs have made the concept of elimination more realistic. In 2005, it was announced that the ministries of Health of India, Bangladesh and Nepal in collaboration with WHO have agreed to start a

cross-border programme to eliminate the threat of VL by the year 2015. This certainly shows a greater political will and commitment, but large-scale international funding will be needed to support the local governments to implement the plan (WHO 2005).

Antimonials and Visceral Leishmaniasis

Antimony (Sb), as the related element arsenic (As), is a metalloids belonging to the group XV of the periodic table of elements. These metals exist in various forms, all exhibiting different biological properties and thus having varying toxicity. The trivalent forms of these metals mediate high toxicological effects which seem to be mainly due to their oxidative behaviour.

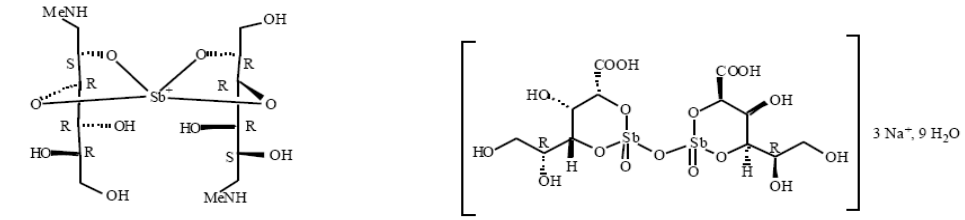


Fig 3.2: Structure of antimonials

Antimonials have been known since ancient times as an eye cosmetic but also as a medicine. Pliny wrote of 7 medicinal remedies in the 1st century B.C. using stibium or antimony sulphide, and for centuries it was prescribed for a variety of ailments. In the beginning of the 20th century, tartar emetic, an organic trivalent form of antimony, was used for the treatment of sleeping sickness; and Gaspar Vianna introduced the drug for the treatment of Mucocutaneous Leishmaniasis in 1912. The activity of antimonials against the various clinical forms of leishmaniasis was soon discovered in Italy and India. Since tartar emetic was highly toxic for humans, new less toxic and more effective antimonials were

developed and evaluated. In India Brahmachari made Ureastibamine@, which was probably the most used of this class of compound. The pentavalent antimony (SbV) salts were found to be 10-fold less toxic to mammals than the trivalent (SbIII) salts. By the end 1940s, this pharmaceutical research produced the current drugs in use: the closely related organic pentavalent antimonial compounds sodiumstibogluconate (SSG) and meglumine antimoniate (Goodwin, 1995).

In vivo pharmacokinetics

The kinetics of intramuscular administered pentavalent antimonials has been described as a three term kinetic model *in vivo* (Chulay, 1988):

1st term: Initial absorption phase with a half life of about 0.85 hours.

Both formulations are rapidly absorbed in the first central compartment (blood and plasma), resulting in peak blood SbV concentration of about 12-15 mg/L at about 2 hours after treatment.

2nd term: Rapid elimination phase with a half life of about 2 hours.

More than 80% of the administered drug is eliminated from the central compartment via the urine in 6-8 hours after treatment. After about 16 hours, drug blood levels fall to less than 1 % of the peak levels. Conversion to trivalent antimony during this rapid phase, if any, is minimal.

3rd term: Terminal slow elimination phase with a half life of about 76 hours.

This term coincides with the slow urinary excretion of SbIII, and therefore is probably related to the *in vivo* conversion of SbV to SbIII in other peripheral compartments. The long half- life of this 3rd implies that steady-state equilibrium is only reached after minimum 10 days treatment.

The minor amounts of drug absorbed by the organs from the blood in the short time frame of peak-levels accounts for the therapeutic effect of pentavalent antimonials. There are no reports of Sb concentration in human organs during treatment following the current standard regimens. In hamsters, it was found that Sb distributes well to both skin and viscera which explains why antimonials are effective against the different clinical forms of leishmaniasis. Furthermore, the transient peak of Sb concentration in blood might be an underestimate of the Sb concentrations to which amastigotes within macrophages are actually exposed, as *in vitro* mΦ work suggests that SbV could be accumulated by the host cell. This also implies that the duration of drug exposure to the parasite is reflected in the 76 hours long half-life of the last kinetic term (Goodwin, 1995; Roberts et al., 1995).

Leishmanicidal activity of SSG, SbV and SbIII

The intrinsic susceptibility of *Leishmania* to SSG varies between different species. *In vitro* studies using mΦ-amastigote models have shown that *L. (L.) donovani* is 3-5 fold more susceptible to SSG than *L. (L.) major*, *L. (L.) tropica* and *L. (L.) mexicana* (Allen and Neal, 1989). Likewise, the susceptibility for SSG varies in different life stages. For *L. (L.) donovani*, promastigotes tolerate higher concentrations than intracellular amastigotes. The reduced form SbIII on the other hand, is highly toxic for both life stages. This Sb-activity pattern reflects the hypothetical dual mode of action of SSG discussed

below: (i) upon contact with infected mΦ, SSG helps the mΦ to kill its intracellular guests and when reaching the parasite, SbV is reduced to SbIII; (ii) SbIII can further directly kill the parasite.

Interaction of SSG and host cell

The interaction between SSG and the infected mΦ has received little attention in *Leishmania* studies on antimonials, although there always has been clear evidence pointing out the importance of the mΦ for SSG activity. First of all, SSG can kill amastigotes living within mΦ, but is much less effective against axenic amastigotes (e.g. cultured without mΦ) (Roberts et al., 1995; Ephros et al., 1997) and secondly is much less effective in immune-compromised hosts (Fernandez-Guerrero et al., 1987; Escobar, 2001). Together, these observations imply that SSG requires mΦ in a functional immune-system to fully unfold its activity. *L. (L.) donovani* modulates the signalling pathways of it's host cell extensively in favours of its own survival. Recent research now suggests that SSG modulates the signalling of the mΦ as well, thereby restoring the mΦ's protective mechanisms against the parasite.

SSG triggers Reactive Nitrogen Intermediates (RNI)/Reactive Oxygen Intermediates (ROI) generation

In vitro treatment with 10µg/mL SSG (comparable to drug concentrations in blood) of uninfected mΦ induces the generation of both ROI and RNI. ROI production is immediate and already peaks at 3 hrs after treatment and is fading out 3hrs later, this effect has also been observed in the whole blood of animals after SSG treatment (Rais et al.,

2000)**.** RNI generation on the other hand, gradually builds up in the first 24 hrs after SSG treatment and maintains a high level for at least another 24 hrs**.** A similar ROI/RNI pattern is observed upon SSG exposure of *L (L.) donovani* infected mΦ. The respective peak levels of ROI/RNI generation coincide with peak leishmanicidal activity. Upon scavenging of ROI/RNI or in absence of iNOS (mΦ from iNOS KO mice) SSG fails to kill amastigotes in the first 48 hrs after treatment. This suggests that *in vitro*, during the first 48 hrs, SSG does not kill the parasite directly, but indirectly through triggering ROI/RNI production in the mΦ (Mookerjee et al., 2006).

SSG triggers production of Cytokines

SSG treatment of infected or uninfected mΦ also induces expression of IL-12 (Mookerjee et al., 2006). IL-12 plays a central role in the development of a specific T-cell response crucial for control of *Leishmania* infection. However, in active visceral disease, this T-cell response is futile due to the suppressive action of omnipresent IL-10. SSG treatment probably helps reversing this suppressive state by inducing IL-12 expression which would tip the balance back in favour of a pro-inflammatory response.

The specific T-cell response acts mainly through IFN-γ which activates the infected mΦ, but *L. (L.) donovani* paralyses IFN-γ responsiveness of its host cell through SHP-1. Thus, SSG, as potent inhibitor of SHP-1, could restore IFN-γ responsiveness and thereby switch on the leishmanicidal activity of the mΦ. *In vitro* studies already demonstrated increased JAK/STAT phosphorylation (signalling downstream IFN-γ) upon SSG treatment (Pathak and Yi, 2001). In addition, the expression of the IFN-γ-receptor is also up regulated in response to SSG treatment, which could further contribute to the IFN-γ responsiveness

of the mΦ (Dasgupta et al., 2003). This synergy between SSG and the host immune system could play an important role in the pharmacological effect of SSG in VL treatment. This hypothesis is also supported on clinical level as SSG is ineffective in curing VL in immune-suppressed patients; thus it seems that SSG cannot beat *Leishmania in vivo* in the absence of a functioning immune system.

Interaction of SbV/SbIII with *Leishmania*

The second panel of SSG action against *Leishmania* comprises the direct leishmanicidal activity of SbIII. SbV is relatively inert and exerts minimal direct toxic activity against eukaryotic cells. However, the drug is metabolically converted *in vivo* and *in vitro* to SbIII, which is highly toxic to both mΦ and amastigotes, through its oxidative properties, binding functional thiol-groups in proteins and peptides.

Uptake of SbV and SbIII by *Leishmania*

The uptake route of SbV has not been identified yet. Possibly, the uptake of SbV is mediated by a transporter recognising a sugar-like structure resembling the gluconate portion of SSG, as gluconate does inhibit SSG uptake in axenic amastigotes. Amastigotes can accumulate significantly higher amounts of SbV compared to promastigotes, which probably also contributes to the amastigotes-specific activity of SbV.

SbIII, like AsIII, enters bacterial, yeast and human cells through aquaglyceroporins (AQP). AQPs belong to the major intrinsic protein family; they are integral membrane channels that transport water and other uncharged polar solutes such as glycerol and Urea bidirectionally. Several genes encoding AQPs have been identified in the

sequenced genomes of kinetoplastids. AQP1 is currently considered as the main uptake route of SbIII by the parasite during SSG treatment (Gourbal et al., 2004).

Reduction of SbV to SbIII

The reduction of SbV to SbIII is tricky to characterise as both the parasite and the mΦ could participate and several mechanism might be involved. Very likely, this process of reduction varies between different species and depends on the mΦ-system used; which complicates the matter further and hampers comparison of various studies.

On the level of the macrophage, it was recently proven that the toxicity profiles of SbV and SbIII are unrelated. SbIII is highly toxic to mΦ, while SbV is well tolerated. But most importantly, there is no trace of the typical SbIII-like mediated toxic effects (e.g. GSH efflux, loss of redox potential, apoptosis) upon SbV exposure suggesting that SbV is not converted to SbIII in the macrophage (Wyllie and Fairlamb , 2006.)

The mechanism of SbV reduction in amastigotes could be enzymatic or non enzymatic. The thiols, glutathione and trypanothione, have both been reported to spontaneously reduce SbV to SbIII under acidic conditions (Ferreira Cdos et al., 2003). However, the reported rates of spontaneous conversion *in vitro* are relatively low and slow, which questions the physiological importance of this reaction inside parasites with an neutral intracellular pH (Gebre-Hiwot et al., 1992). Furthermore, promastigotes also take up SbV and have higher trypanothione and glutathione concentrations than amastigotes (Wyllie et al., 2004). Thus if such a spontaneous reduction could take place in the parasite, one would expect that promastigotes can also reduce SbV, but this was proven not to be the

case in *L. (L.) donovani* (Shaked-Mishan et al., 2001). It cannot be excluded though that such a spontaneous SbV reduction does play a role in the parasitophorous vacuole, where SbV possibly accumulates in acidic conditions, thus favouring the spontaneous reaction. The SbV reduction mediated by the amastigotes themselves is more likely to be catalyzed by an enzyme. Two potential *Leishmania* reducing enzymes have been identified, a thiol-dependent reductase (TDR1) (Denton et al., 2004.) and an arsenate reductase (ACR2). TDR1 is related to the mammalian glutathione S-transferase and the *L. (L.) major* recombinant enzyme was proven to reduce SbV to SbIII using glutathione in vitro. TDR1 has a 10 fold higher expression in intracellular amastigotes compared to promastigotes which is consistent with the observed stage-specificity of SbV reduction (Denton et al., 2004). The second enzyme, ACR2 is a homologue of a glutaredoxin-dependent yeast arsenate reductase which reduces AsV to AsIII both *in vivo* and *in vitro* (Mukhopadhyay and Rosen, 1998). The *L. (L.) major* form of ACR2 reduces SbV to SbIII in vitro in the presence of glutathione and glutaredoxin. Transfection of ACR2 in *L. (L.) infantum* resulted in increased SbV sensitivity of the corresponding intracellular amastigotes, which supports an *in vivo* role of ACR2 in the amstigote mediated SbV reduction. A recent study has demonstrated that the actual physiological role of ACR2 in *Leishmania* possibly lies in protein tyrosine dephosphorylation (Zhou et al., 2006).

SbIII mechanism of action on *Leishmania*

SbIII's fatality for *Leishmania* primarily lies within its interaction with sulfhydryl-groups which severely affects the protective thiol metabolism of the parasite at 2 levels:

(1) **SbIII- mediated thiol efflux:** *In vitro* exposure of either promastigotes or amastigotes to SbIII is rapidly followed by a time and dose- dependent specific efflux of GSH and $T(SH)_2$ in equimolar amounts. This thiol-efflux pattern is most likely a reflection of the parasite's effort to detoxify SbIII by exporting the drug as a stable complex with endogenous thiols. SbIII in particular can form a stable complex with GSH and $T[SH]_2$, (GSH) SbIII ($T[SH]_2$), as demonstrated by electrospray ionisation-mass spectrometry. The metal-thiol efflux pump responsible for this detoxification remains elusive, although its existence could be experimentally confirmed in several *Leishmania* species, including *L. (L.) infantum*. In mammalian cells, thiol-metal efflux is mediated by ABC-transporters of the MRP family. The *Leishmania* genome has revealed several homologues belonging to the MRP-family. One such homologue, MRPA, has already been studied extensively with respect to its role in metal-thiol transport. But, MRPA's location in intracellular vehicles excludes this pump from being involved in the rapid SbIII-mediated thiol disappearance.

(2) **SbIII inhibition of trypanothione reductase (TR):** SbIII, not SbV, has been shown to be an inhibitor of trypanothione reductase (TR) *in vitro*. The inhibition is probably due to the formation of a covalent bond between SbIII and the reduced cysteine residues in TR's active site. The parasite's continuous oxidative metabolism results in formation of GSSG and TS_2, which under normal circumstances would be reduced by TR in order to maintain the redox potential. However, in the presence of SbIII, TR activity is impaired and thus leads to continuous accumulation of dithiols in SbIII treated *L.(L.) donovani*.

The combined effects of these 2 events perturb the redox state of the parasite. The redox potential of any cell correlates with the biological status of the cell. A low redox potential signifies that a high number of electrons are available to use in the various oxidation processes linked to building cellular components and oxidative stress management; therefore low redox potentials are related to cell proliferation. As the redox potential increases, the cell loses its capacity to fuel biosynthetic reactions and more importantly for a parasite, to protect against oxidative stress. Consequently high redox potentials are generally related to apoptosis. In *Leishmania*, the redox potential is mainly determined by the 2 redox couples $TS_2/T(SH)_2$ and GSSG/2GSH. The efflux of both $T(SH)_2$ and GSH and the accumulation of TS_2 and GSSG upon SbIII exposure as described above , leads to a substantial 'loss of available electrons' or increase in redox potential of both couples. This makes the parasite extremely vulnerable to oxidative stress, especially since exactly at the same time its host cell is also responding to the drug by fully unfolding its oxidative killing mechanisms. The combination of increased redox-potential (by SbIII on the parasite) and increased oxidative stress (by SSG on the mΦ) indeed drives the amastigotes towards an apoptotic- like state characterised by loss of mitochondrial membrane potential, DNA-fragmentation and externalisation of phosphatidylserine in the plasma membrane. This apoptotic- like fits the life-style of *Leishmania* as it would prevent the release of harmful products that could adversely affect the remaining surviving residents in the parasitophorous vacuole.

SSG treatment failure and antimony resistance in *Leishmania*

Treatment failure can be due to many causes and can range from never showing any response, to relapse several months after an apparent clinical cure. These two extremes, complete unresponsiveness or relapse, have different clinical and/or biological implications. Drug resistance on the other hand refers specifically to the parasite's susceptibility and is only one of the possible causes of treatment failure.

Table 3.1: Potential causes of treatment failure

Treatment Related	Parasites Related	Host Related
• Drug quality • Treatment compliance • Dosage	• Intrinsic insensitivity of species • Naturally emerged drug resistance	• Clinical presentation • Characteristics of patients • Immunological response • Host genetics

Relapse in VL

Relapse of VL after a successful treatment with any drug regimen is well recognised and affects about 5-10% of apparently cured patients. The risk of relapse is highest in the first 6-12 months after treatment, thus clinician only consider patients definitely cured if this post-treatment period passes uneventful (Murray, 2004**.**). The reason for risk of relapse after successful treatment lies in the fact that apparently no treatment can eliminate all parasites from all tissues, not in VL or any other form of Leishmaniasis(Murray et al.,1996 ; Belkaid et al., 2001). In other words, treatment does

not result in sterile cure, but patients presenting with clinical cure presumably still harbour quiescent parasites, probably for life. A similar phenomenon is seen in asymptomatic individuals who are chronically infected but do not show any signs of disease.

Resistance to SSG in North Bihar

Pentavalent antimonials currently have an efficacy around 90 % for the treatment of VL almost all over the world, except in North Bihar, India. The state Bihar is situated in the east of India, it is a rural poor area where daily family income is no more than 1US $. North Bihar was first reported to have problems of decreasing SSG efficacy during the epidemic in the late 1970s. Since then, SSG treatment regimens were regularly modified by increasing both doses and duration in an effort to counteract the gradual decreasing SSG efficacy. Nevertheless, during the epidemics of the early 1990s, the situation deteriorated dramatically with cure rates plummeting to 30-40%.

A strictly supervised 2-site study was carried out in 1994-1997 in Muzaffarpur (North Bihar) and Varanasi (Uttar Pradesh) which is situated about 300 km west of Muzaffarpur. In Muzaffarpur, 65% of VL patients could not be cured, with 51% of the patients presenting with immediate unresponsiveness after a complete 30 day SSG regimen. These dramatic figures officially confirmed that SSG was no longer useful in North Bihar. Fortunately, the report from Varanasi was far more positive; 86% of the patients had definite cure with SSG, with less than 2 % of the patients showing immediate unresponsiveness. The geographically restricted nature of the SSG problem

implied that some factors specific to North Bihar was the actual undercurrent for this clinical disaster (Sundar et al., 2000).

A study on the use of SSG treatment in Bihar during the epidemic in the early 1990s shows a widespread abuse of the drug (Sundar et al., 1994). The majority of the patients consulted unqualified medical practitioners, who often administer the treatment inappropriately. For example, it is common practice to start with a small dose and gradually build up to the full dose over a week, or to give drug free intervals, or to split the daily dose in two, often with the belief that this might reduce renal toxicity. These practices result in sub-optimal drug concentrations in the patient and could certainly lead to progressive SSG tolerance of the parasite. In addition, the majority of the patients did not comply with the full course treatment. Many patients quitted treatment for economic reasons or simply because they felt better and wanted to go back to school or work. These facts indicate that both doctors and patients in this particular impoverished area were unaware of the importance of the correct use of treatment. The resulting dismal clinical practice was quite likely the major contributor in rendering the only widely available and affordable drug useless (Sundar et al., 1994; Sundar and Murray, 2005).

Natural SSG resistance

The events that took place in North Bihar described above, suggest the emergence of natural SSG resistant parasites. The 20 year struggle with decreasing SSG efficacy was very likely a dynamic process with changing causes of treatment failure. At first, treatment failure was indeed probably the result of administering inadequate doses and lack of treatment compliance, but without loss of SSG effectiveness against the parasite.

However, the suboptimal treatment regimens presumably provoked the gradual selection of SSG resistant *L.donovani*. Hypothetically, in an anthropnotic focus such as Bihar, these SSG resistant parasites could have a selective advantage over sensitive parasites. The selective advantage combined with the intense transmission in Bihar, would lead to a rapidly increasing proportion of patients infected with SSG resistant parasites. The epidemics of the 1990s possibly generated the permissive conditions for the spreading of newly emerged SSG resistant *L.donovani* and this spreading was clinically reflected in the sudden drastic decrease of SSG efficacy seen in the mid 1990s. High rates of immediate SSG unresponsiveness (in contrast to delayed relapse) despite administering complete treatments is typical for infections caused by SSG resistant parasites (Sundar et al., 2000).

Furthermore, the degree of SSG efficacy was reported to vary in the different districts of Bihar. It seems that this geographical variation correlates with the local degree of endemicity. The higher the endemicity degree, and thus transmission rate, the lower SSG efficacy, which tallies with the view that the transmission rate determines the degree of spreading of SSG resistant parasites.

Ultimately, conclusive proof for the emergence of natural SSG resistance can only be attained by demonstrating the presence of natural SSG resistant parasites in patients. Such studies depend on *Leishmania in vitro* SSG susceptibility tests, which are discussed below.

***In vitro* drug susceptibility testing of *L.donovani* isolates; limited choice for SSG.**

Testing the drug susceptibility of *L. (L.) donovani* from patients requires the isolation of these parasites as amastigotes in aspirated or biopsied lesions, usually bone marrow, spleen or lymph nodes. The infected biological material is inoculated in appropriate culture medium in which the intracellular amastigotes will transform to extracellular promastigotes. The obtained promastigotes can then be used directly or indirectly in a variety of in vitro susceptibility tests used for any drug:

(i) **Promastigote model:** Promastigotes are cultured readily in cell free monophasic media at 25-28°C and their drug susceptibility profiles can be determined easily by incubating parallel cultures with varying concentration of the drug. However SSG activity is specific for amastigotes; thus there is no point in a direct susceptibility test on promastigotes to check for natural SSG resistance. Indeed, it has been shown that when testing parasites isolated from patients, the in vitro SSG susceptibility profiles of promastigotes and corresponding intracellular amastigotes do not correlate (Ibrahim et al., 1994; Lira et al., 1999).

(ii) **Axenic amastigote model:** Promastigotes collected from patients can be transformed to axenic amastigotes by reducing the pH of the medium to 5.5-6.5 and elevating the temperature to 37°C. This method was first described for *L. (L.) mexicana* but also exists for *L. (L.) donovani* and *L. (L.) infantum* (Sereno et al., 1998; Ephros et al., 1999). The drug susceptibility test with axenic amastigotes is equally easy as with promastigotes. Although axenic amastigotes seem to be susceptible to SSG in contrast to promastigotes (Sereno et al., 1998), this system does not perform satisfactory to test the presence of

natural SSG resistance in several aspects. First of all, since the development of axenic amastigotes, there has been an open concern whether these are 'real' amastigotes despite their confirming biochemical profile (Sereno et al., 1998). Secondly, the action mode of SSG involves both the host cell and the parasite. Susceptibility profiles of axenic amastigotes would only reflect the effectiveness of part of the SSG activity. Axenic and intracellular amastigotes were indeed proven to have differential SSG susceptibility profiles (Goyard et al., 2003; Wyllie et al., 2004). Thirdly, and most importantly, adaptation of isolated parasites to axenic amastigote cultures is a hard, selective, and lengthy process, which makes this type of assay inapt for high-throughput testing of patient's isolates.

(iii) **Macrophage-amastigote model:** This final model tests the susceptibility of intracellular amastigotes *in vitro* infected macrophage. Typically, amastigote-infected macrophages are maintained in a medium with serial dilutions of drug for 3-7 days at 37°C. Drug activity is then assessed by either determining the percentage of infected macrophage and/or the number of amastigote/ macrophage. These parameters are currently still determined by time-consuming microscopic counting. There have been several attempts to automate this process by using fluorometric methods, but so far these methods have limited success (Neal and Croft, 1984). Two different types of macrophage can be used in this model:

a) Monocytic cell lines: Mouse (J774, RAW264.7) and human (THP-1, U937, HL-60) monocytic cell lines are frequently used to determine SSG susceptibility. Ideally these cells need to be stimulated to differentiate into non-dividing cells before the *in vitro* infection and drug test, otherwise the changing host cell densities (due to division during test) raises problems for evaluation and interpretation of the susceptibility assay.

b) Primary isolated cells: This usually concerns murine peritoneal macrophage or human-monocyte transformed macrophage. The disadvantage of this system is clearly the difficulty to isolate and establish primary cultures, adding extra work and time to an already laborious method. However, in contrast to monocytic cell lines, the amastigote survival can be determined unambiguously as these differentiated cells do not divide.

Although the macrophage-amastigote model is currently the most adequate method available to test SSG susceptibility of *Leishmania* isolates from patients, there are still numerous shortcomings and disadvantages:

(i) The isolation of parasites from the host and transfer to culture medium is bound to involve some selection of subpopulations best adapted for growth on the culture medium, thus the tested isolate is not exactly the parasite population that was present in the patient in the first place (Fumarola et al.,2004).

(ii) *In vitro* susceptibility tests in macrophage-amastigote models do not take the host's immune system into account, while *in vivo* SSG activity presumably largely relies on the immune factors present in active disease. The importance of immune factors in evaluation of SSG susceptibility was emphasized by parallel *in vivo/in vitro* experiments. SSG is ineffective to treat *L.donovani* infected nude and SCID mice, but the same *L. donovani* strains infected *in vitro* in peritoneal macrophage derived from nude or SCID mice are susceptible to SSG. This demonstrates that the *in vitro* SSG susceptibility of *L. donovani* does not necessarily correlate with their *in vivo* SSG susceptibility.

(iii) A last important practical note, different research groups have different protocols for *in vitro* SSG susceptibility testing. Unfortunately, the protocols differ in aspects that

can have a serious influence on the outcome of the susceptibility test such as formulation of SSG used (with or without preservatives), type of macrophage used, level of macrophage infection (higher infections have lower drug activity) (Neal and Croft, 1984; Seifert et al., 2010), time of exposure with SSG and concentration of foetal calf serum. Unfortunately the exact conditions used for in vitro SSG susceptibility testing of isolates are not always mentioned, which makes it impossible to compare such reported screening results.

Features of natural SSG resistant *L.donovani* from North Bihar

The first study undertaken to determine whether natural SSG resistance had emerged in North Bihar tested the *in vitro* susceptibility of 24 isolates (9 from cured patients, 15 from unresponsive patients) in murine peritoneal mΦ. The isolates from SSG cured patients (ED_{50}= 2.4µg/ml) were shown to be threefold more susceptible to SSG compared to the isolates from SSG unresponsive patients (ED_{50} = 7.4µg/ml). The difference between the 2 groups of isolates was significant and thus supported the concept of emergence of natural SSG resistance in India (Lira et al., 1999). Later we also confirmed these findings with *in vitro* susceptibility tests of sizable number of Kala-azar and PKDL isolates. Assessment of the natural susceptibility of PKDL and Kala-azar isolates to Sodium antimony gluconate (SAG) using intracellular amastigotes, gave similar mean ED_{50} for KA and PKDL isolates and a good correlation with clinical response. All KA isolates and a significant proportion of PKDL isolates from high endemicity zones were SAG resistant (Singh et al., 2006). Further, Indian *Leishmania donovani* isolates from zones of varying antimony resistance displayed significantly

correlated *in vitro* susceptibility towards antileishmanial drugs: SAG, AmphotericinB and Miltefosine, raising the possibility of cross-resistance mechanisms operating in the field isolates (Kumar et al., 2009).

Gaps in existing Research:

Studies towards understanding the mechanism of antimony resistance have been mostly confined to lab generated resistance. So far, very few studies have been carried out to characterize the status of drug resistance in field isolates. This study will provide the picture of global gene expression differences in drug resistant and sensitive parasites. The success of this work depends on an experimental approach, the microarray, which takes full advantage of the resources available in the post-genomic era. The application of this technology to study gene expression in *Leishmania* is well established in our lab. Eradication of Leishmaniasis has been identified as a goal of national importance. This study based on field isolates will lead to an understanding of the key genes in the parasite that are involved in antimony resistance.

Introduction

The *Leishmania* parasite is transmitted by an invertebrate sandfly vector, *Phlebotomus argentipes*. The parasite leads a digenetic life cycle with two main stages: the flagellated promastigotes, which are found in the gut of the insect vector, and the intracellular amastigotes, which live inside macrophages of the mammalian host. Infected sandflies introduce the metacyclic forms of the promastigote stage into the bloodstream of the vertebrate host when they bite to take a meal. The promastigotes transform into an amastigote stage in the phagolysosome of reticuloendothelial cells, where they multiply in the hostile environment of the macrophage and kill the cells.

In the absence of effective vaccine, chemotherapy is the main weapon to control infections. The first line treatment for Visceral leishmaniasis (VL) is a pentavalent antimony (SbV) such as sodium antimony gluconate (SAG) or meglumine antimoniate as recommended by the World Health Organization (WHO,1990), despite its requirement for long courses and cardiac toxicity. In the Indian sub-continent, the efficacy of SAG has gradually declined in spite of regularly increasing both doses and duration of treatment. However, there is a geographical variability in efficacy even within this region (Lira et al., 1999; Singh et al., 2006). Similarly, in South East Nepal, the average efficacy of antimonial treatment was reported to be 90% between 1999 and 2000, but this value dropped to 76 % in Nepalese districts bordering Bihar(Rijal et al., 2003). In India, as many as 65% of previously untreated patients fail to respond promptly or relapse after therapy with antimony drugs, due to the development of drug resistance (Sundar, 2001a). Amphotericin B (AmB) is used as an alternative, although it is a powerful antileishmanial agent this drug remains a second line of defense because of its high cost and

severe toxicity. Recently, miltefosine (MLF), a compound originally developed as an antitumour agent, has been shown to be an orally effective drug against kala-azar (Sundar et al., 2002). A regimen of 100 mg per day or 50 mg twice a day for 3-4 weeks was observed to produce a cure rate of 100%, but its long half life may lead to resistance. Therefore monitoring and prevention of resistance towards antimonials has become a priority. Within this context an insight into the mechanism of drug resistance could contribute to the development of efficient strategies for monitoring antimony resistance at sites where it is endemic.

Drug resistance is a complex phenomenon in the *Leishmania* parasite as several metabolic pathways and membrane transporters are implicated in the resistance phenotype. Different mechanisms for drug resistance have been suggested such as gene amplification and the parasite's inability to convert SbV to SbIII (Ouellette et al., 2004; Croft et al., 2006). A targeted DNA microarray with 44 known genes known to be responsible for resistance has been used to show the linkage of the genes to drug resistance in the *Leishmania* parasite (Guimond et al., 2003).

Molecular detection tools like microarray technology have been employed to identify novel drug targets to overcome this situation and provide a more rapid and high throughput alternative for elucidation of the mechanisms leading to resistance. Various molecules like multi drug resistance protein (MRPA), nucleoside transporter, long chain fatty acid CoA ligase, parasite surface antigen-2, along with many hypothetical proteins (Salotra et al., 2005; Singh et al.,2007; Leprohon et al., 2009) have been identified using these methods. Recently, using transcriptome profiling, differentially expressed genes in antimony resistant *L. infantum* were found to be physically linked in the genome and the correlation of antimony resistance levels and

the copy number of aneuploid chromosomes suggest a putative link between aneuploidy and drug resistance (Leprohon et al.,2009). The available information on antimony resistance indicates that several mechanisms may coexist in the same cell and different mechanisms may operate in field isolates compared to lab generated resistant parasites (Singh et al., 2003; Croft et al., 2006). Here, we exploited genomic microarray for evaluation of *Leishmania* gene expression between antimony sensitive vs resistant strains isolated from kala azar patients. The genes showing altered expression were validated by RT-PCR and western blotting.

Materials and Methods

1. Reagents and Chemicals

Agarose, Bovine Serum Albumin, Glycine, Glutamine, HEPES, Penicillin, Streptomycin, Gentamycin sulphate, Tris base, Medium-199, and other fine chemicals for microarray experiments i.e. yeast tRNA, aminoallyl-dUTP and Poly A were from Sigma Chemicals, USA. Trizol, *Taq* polymerase, dNTPs, $MgCl_2$, DTT, Superscript II reverse transcriptase, oligo dT_{20}, Cot-1 DNA, RNasin were from Invitrogen, USA. Fetal bovine serum was obtained from Biological Industries, Israel and Gibco BRL, USA. Cy3-monofunctional dye or Cy5-monofunctional dye for labeling in microarray experiments were from Amersham Biosciences, USA. SpotReport® Alien cDNA array validation system (alien mRNA spikes 1-3) was from Stratagene. Kits for gel extraction, PCR purification and RNA cleanup (RNeasy kit) were obtained from Qiagen. RNA marker was from MBI Fermentas & New England Biolabs, USA. Gene specific oligonucleotides were synthesized from IDT, USA. Disodium hydrogen phosphate, Dipotassium hydrogen phosphate, Isopropanol, Potassium dihydrogen phosphate, Potassium chloride, Potassium acetate, SDS, Sodium acetate, Sodium chloride, Sodium dihydrogen phosphate, Sodium hydroxide, Sodium bicarbonate were purchased from SRL, India. Magnesium sulphate, Glucose was from Qualigens, India.

2. Patients and *Leishmania donovani* Isolates

Nineteen *L. donovani* isolates were prepared from bone-marrow aspirates of VL patients, as described elsewhere (Sreenivas et al., 2004). The patients originated from Bihar and West Bengal with zones of low and high endemicity for VL, which represent, respectively, zones of low resistance (LR) and high resistance (HR) to antimony (Sundar et al., 2001b). All patients

received either 20 mg SAG /kg/ day intramuscularly for 30 days or AmB treatment (infusions of 1 mg/kg on alternate days for 1 month. Informed consent based on the guidelines of the Ethical Committee, Safdarjung Hospital, New Delhi, was obtained from the patients. The bone marrow samples from VL patients were aseptically collected in medium M199 pH 7.4, and 25mM HEPES supplemented with 20% FBS with 100µg/ml streptomycin and 100U/ml penicillin and incubated at 24°C. Promastigotes were cultured at 24°C in M199 medium with 25mM HEPES (pH7.4) supplemented with 10% FBS, 100 IU and 100 µg/ml each of penicillin G and streptomycin, respectively and drug susceptibility of the parasite isolates were determined in less than 6 passage after isolation from patients (Sreenivas et al., 2004; Singh et al., 2006). Stationary stage promastigotes were used for infection of macrophages to mimic the situation in humans.

Susceptibility of intracellular amastigotes to antileishmanial drugs

An intracellular assay for *L. donovani* was performed as described elsewhere (Singh et al., 2006). Murine macrophage–adherent cell line J774A.1 ($2X10^5$ cells/well) in 8-well chamber slides were infected with stationary-stage promastigotes at a 10:1 (parasite**:** macrophage) ratio and were incubated in 5% CO_2 for 4 h at 37ºC. Unattached promastigotes were washed off, and the cells were incubated for 18–24 h. Infected cells were re-incubated, for 48 h, with different concentrations of SAG - 3, 10, 30, 60, and 100 µg/ml, while DMEM with 10% FBS was added as controls. After staining with Diff-Quik solutions, the numbers of *L. donovani* amastigotes per cell were microscopically counted at X100 magnification in 100 macrophages. All the concentrations were tested in duplicates and the assay for each isolate was performed in triplicate. The percent killing was calculated by use of sigmoidal regression analysis (Origin 6.0;

Origin Lab), and the ED_{50} were determined. The ED_{50} calculated were revalidated by repeating the drug assay experiment with the ED_{50} concentration obtained by sigmoidal regression analysis.

3. Generation of antimony resistant *L. donovani* (K80SbIII)

Two clinical isolates of *L.donovani*, K80 and K135 were used for the microarray study on the basis of their antimony susceptibility. K80 was adapted as SbIII resistant parasite by *in vitro* passages with a stepwise increase in the concentration of SbIII from 10 to 125 µg/ml(K80SbIII) in the Medium M199 + 10 % FBS. Drug concentration was increased only when the drug exposed parasites showed a growth rate equivalent to that of the parallel growing wild-type parasites. K135 was used as a sensitive isolate.

4. **Isolation of RNA**

To understand the pattern of gene expression in SAG sensitive and resistant isolates of *L. donovani*, we used RNA isolated from K135 and K80SbIII *Leishmania* parasites respectively. The antimony sensitive and resistant cells were collected at identical growth points and grown under identical conditions to minimize variations in the quality of RNA. Total RNA was isolated from promastigotes using Trizol reagent. Briefly, the cells ($1\text{-}1.5X10^9$) were lysed in Trizol, at room temperature and to the homogenized samples, 0.2 volumes of chloroform was added followed by centrifugation at 12,000 x g for 15 min. The aqueous phase was collected and 0.5 volumes of isopropyl alcohol were added to precipitate total RNA. The total RNA was pelleted by centrifugation and washed in 70% ethanol (in autoclaved DEPC treated water) and stored at –70°C till further use. Approximately 130-140µg of the total RNA was cleaned up to eliminate

salts and contaminating genomic DNA by preferential binding of RNA to the RNeasy columns before the labeling reaction, using RNeasy mini kit. The purified RNA was quantified spectrophotometrically by checking the absorbance at 260nm. The quality and integrity of RNA was checked using agarose/formaldehyde gel electrophoresis. To 1µg RNA, 0.5µl 10X MOPS buffer, 4µl (12.3 M) formaldehyde, 4µl formamide and 0.5µl ethidium bromide were added. The samples were heat denatured at 66^{o}C for 10 mins, mixed with 2X RNA loading buffer and loaded onto the gel. Electrophoresis was carried out at 65V for 3hrs and products visualized under UV transilluminator on 0.6% Formaldehyde Agarose Gel.

6. **Microarray hybridization, Scanning and Analysis**

A. Description of *L. donovani* Genomic Microarray

The *L. donovani* genomic microarray used in this study was constructed as a part of a collaborative project with Dr. H.L. Nakhasi and Dr. R. Duncan at CBER, FDA, USA. Briefly, a library of 1-1.5 kb randomly sheared fragments of genomic DNA from a fresh isolate of *L. donovani* prepared from an Indian KA patient was ligated into the pZERO-2 vector. A total of 8448 PCR amplified inserts from library clones, representing ~60% of the expressed genes, along with alien external DNA, 24 known *Leishmania* genes viz. 28SrRNAζε, *Leishmania* protein kinase 2 (c-lpk2), *Leishmania* stress inducible protein (LSIP), LdS-6-1, Calreticulin, Centrin, protein disulfide isomerase (PDI), Protein kinase A (PKA) etc. and 12 negative controls inclusive of human genes, bacterial and salmon genomic DNA, hamster genes, empty vector, poly A, cot-1 etc, were printed in duplicate on poly-lysine slides.

B. Microarray Hybridization and analysis

i. Production of differential probes

Fluorescently labeled cDNA copies of the total RNA isolated from log phase sensitive and resistant parasites were prepared that represent all the genes expressed. Alien RNAs that are complementary to the Alien external control DNAs printed on the slide were also included in the labeling reaction to facilitate validation and optimization of hybridization reactions. cDNA was prepared by using 20µg of total RNA spiked with alien RNAs. 1µg oligo $(dT)_{20}$ primer, 10mM each of dATP, dCTP and dGTP, 6mM of dTTP, 4mM of amino-allyl-dUTP, 9mM DTT and 400 units Superscript II reverse transcriptase in reaction buffer provided. The RNA and primer were incubated at 70^0C for 5 mins and snap-chilled on ice, before other components of the reaction were assembled. The reaction was incubated at 42^0C for 1hr. The residual RNA in the reaction tube was then degraded and reaction neutralized by keeping the tube at 65 °C for 5 min. The cDNA products were purified using Qiagen MiniElute PCR purification kit and concentrated using DNA plus vacuum concentrators. A cDNA probe from sensitive parasites sample labeled with one fluorochrome was mixed with a probe from resistant parasites sample labeled with a contrasting fluorochrome and hybridized to the microarray, using hybridization chambers.

ii. **Sensitive vs Resistant Hybridization**

The two probes, sensitive and resistant labeled with respective fluorochrome were mixed and hybridized with the genomic microarray chip containing 8448 clones in a solution containing 3.5X SSC, 0.3% SDS, 10µg COT-1 DNA, 4µg yeast t-RNA, 10µg poly A in a hybridization chamber. The hybridization reaction was allowed to proceed for 16hrs at 65^0C.The hybridized microarrays were washed at room temperature for two minutes in 2X SSC/0.1% SDS, and 1X

SSC for 10 mins, 0.2X SSC for 10 mins and finally 0.05X SSC for 10mins. All the hybridizations were carried out with three biological preparations of *L. donovani*.

iii. Scanning and Analysis of microarray data

After hybridization, scanning and analysis of the chips were carried out. The arrays were spun dried in a centrifuge and scanned in a microarray scanner (Axon 4100A). The intensity of the two signals at each spot was transferred directly to a computer for visualization and analysis. The images were visualized using GenePix Pro5.0 software. The slide was initially pre scanned at pixel size 40μm. The fluorescence intensities were calculated for control features and the PMT voltages were adjusted to yield an intensity ratio of 1. Data scan was carried out at a resolution of 5μm, after adjusting the PMT voltages. The individual features on the microarray were manually examined to assess their quality and those exhibiting a sum of Cy3 and Cy5 intensity value lower than the control features included on the array (< 1500) or those having saturation intensity or poor quality were flagged and discarded from further analysis. Local background was subtracted from the intensity value of each feature on the array. The relative intensity of the two signals identified, clones expressed differentially at resistant in comparison with sensitive. Analysis was carried out using statistical analysis software package Acuity 3.1 and MS Excel. The microarray data were normalized based on medians of ratios as well as by Z score transformation as described by Cheadle *et al.,* [2003]. In order to compare expression levels between RNAs, the medians of Cy5/Cy3 ratios were calculated from the normalized values of replicate arrays. The ratios from reverse-labeled experiments were reciprocated before analysis. Clones showing consistent ratios in replicate experiments were selected for further analysis. These fold changes were relative to the amount of RNA in the cell. For calculation of Z ratios of dye flip

experiments, the ratios were inversed before analysis. Z-scores were calculated by subtracting the average gene intensity within one array replicate from the trimmed mean scaled intensity and then dividing the result by the standard deviation of all intensities. This step corrects the data internally in each hybridization and expresses the intensity values as units of standard deviation away from the mean of the array, which is set to zero, while still reflecting the quantitative integrity of the data. Z-ratios were calculated by taking the difference of the average of the replicate Z-scores between two samples and then dividing by the standard deviation of all the averaged Z-score differences for that particular comparison. Average *Z*-scores were also subjected to sample comparisons by grouping the replicates by sample and then applying the *t*-test comparison measure. Each comparison produced a single *p*-value for each gene.

RT-PCR (Reverse transcriptase) analysis

Primers for RT-PCR to amplify the ORF regions of selected genes were designed using Clone 2 or Primer 3 softwares and synthesized from IDT USA (Table 4.1). First strand cDNA was prepared for RT- PCR using 5 µg total RNA and 1µg oligo $(dT)_{20}$ primer. The RNA and primer were incubated at 70^0C for 5 mins and snap-chilled on ice, before other components of the reaction were assembled. 10mM each of dATP, dCTP, dTTP and dGTP, 10mM DTT and 200 units Superscript II reverse transcriptase were added in the provided reaction buffer. The reaction was incubated at 42^0C for 1hr. To check the reaction variability, pUC plasmid (1pg/µl) was spiked in the cDNA preparation in equal ratio. Each reaction was amplified using gene specific primers as well as M13 forward and reverse primers (to amplify 100 bp sequence from pUC plasmid). The reaction mixture (20 µL) for PCR contained normalized cDNA, 200 mmol/L each dNTP, 1.5 mmol/L $MgCl_2$, 25 pmol of each primer, and 0.5 U of Thermus aquaticus (Taq)

DNA polymerase in PCR buffer (Invitrogen, USA). PCR was performed in a DNA thermal cycler (Applied Biosystem, USA). After the PCR amplification, 2 µL of tracking dye was added to the sample, and 10µL of amplification products were run on 1.5% agarose gel containing ethidium bromide (1 mg/mL) in TAE buffer (0.04 mol/L Tris acetate, 0.001 mol/L EDTA). A 1-kb DNA ladder was used as a molecular marker. Control experiments were performed with amplification using total RNA but no reverse transcriptase to check for DNA contamination.

Table 4.1: List of genes and primer sequences used for RT-PCR study

Gene / Clone	Primer sequence	Amplicon size (bp)
Histone 1	F 5'-CTC GCC GCA GAA GTC TCC -3' R 5'-TTC TTC GCC GAG GAT TTC-3'	206
Histone H2A	F 5'-CAG CCG TGC TGG AGT ACC TG-3' R 5'-TGT CGC TTG CCC TTC TTG-3'	233
Histone H4	F 5'-ATG GCC AAG GGC AAG CGC CT-3' R 5'-CGC CGT CAC CGT CTT CTT G-3'	246
MAPK	F- 5'-GGT GTG TGA TTG GGG AGA TGC-3' R- 5'-TCG CCT CGC TAT CCT TCA GG-3'	219
58A8	F 5'-AGC ACA TGC AGG AGC TGT GG-3' R 5'-CTT GAA CTC AGC GTA CTG CGG-3'	228
87B9	F 5'-GAT TAC TGG GGC GAC AAC TAC G-3' R 5'-TCT TCA GCG GCT CGT GAC C-3'	193
42G8	F 5'-AAC GCC TCT GGT CTT GTT ATG-3' R 5'-GCC GTC AGC ACA TCC TTC AC-3'	236
M 13	F 5'-TGT AAA ACG ACG GCC AGT-3' R 5'- CAG GAA ACA GCT ATG ACC-3'	100

7. Western blot analysis

The cell lysates (100µg) from drug resistant (K80 SbIII) and sensitive (K135) *L.donovani* were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membrane strips were blocked, incubated sequentially with the primary antibodies and subsequently with anti rabbit IgG or anti mouse IgG conjugated with HRP. The blots were developed using ECL reagent and visualized on X-ray film. The images were scanned and quantitative assessment carried out with "Image J Software" (NIH IMAGE).

Results

In vitro susceptibility of field isolates to SAG and correlation with clinical response

The susceptibility of field isolates towards SAG was determined at intracellular stage and ED_{50} varied from 2.14 ± 0.28 (mean± SD) to 20.3 ± 0.84μg/ml with mean ED_{50} of 12.18±5.68 μg/ml. A total of 7 out of 19 patients (2 from HR zone and 5 from LR zone) were treated with SAG, both the HR zone patients were non responsive and their respective isolates were found to be resistant in *in vitro* assay. Based on a recent comprehensive study on the SAG pharmcokinetics in Indian KA patients by Thakur et al., (2004), we categorized the parasites with ED_{50} >11μg/ml as resistant. Four of the five LR zone patients responded well to treatment and their respective isolates were found susceptible to SAG in *in vitro* assay while the parasite isolated from the nonresponsive patient (K80) in LR zone displayed high ED_{50} for SAG. Overall the results indicated a strong correlation of clinical response with results of *in vitro* susceptibility assay (Table 4.2).

Table 4.2: Clinical profile of visceral leishmaniasis patients from LR and HR region and *in vitro* intracellular SAG susceptibility of *Leishmania* field isolates.

S.N.	ID	Sex/ Age	Area/Endemicity Region[a]	Treatment/ Response[b]	SAG[c] (ED_{50}µg/ml).
1.	*Ld*AG83		Standard Indian Strain for *L.donovani*		2.06± 0.23
2.	K59	F/21	Vaishali/ HR	SAG (NR)[d]	14.66 ± 3.29
3.	K131	M/22	Saharsha/HR	SAG (NR)	19.38 ± 1.68
4.	K149	M/20	Saran/HR	AmB(R)	15.70 ± 4.01
5.	K192	M/24	Saran/ HR	AmB (R)	20.30 ± 0.84
6.	K251	M/11	Saran/ HR	ND	11.82 ± 1.28
7.	K417	F/8	Muzaffarpur/HR	AmB(R)	14.65 ± 0.67
8.	K429	M/26	Saharsha/HR	AmB(R)	13.76 ± 0.82
9.	K435	M/17	Kushinagar/LR	AmB (R)	11.82±1.39
10.	K439	M/16	Muzaffarpur/HR	AmB(R)	12.88 ± 0.12
11.	K481	M/32	Muzaffarpur/HR	AmB(R)	17.53 ± 0.34
12.	K498	F/55	Madhubani/HR	AmB(R)	15.82 ± 0.24
13.	K516	F/60	Motihari/HR	AmB(R)	16.48 ± 0.61
14.	K509	F/4	Madhubani/HR	AmB (R)	16.84 ± 0.26
15.	K80	F/40	Bhagalpur/ LR	SAG/ (NR) Pentamidine	10.42 ± 2.17
16.	K111	F/36	Siwan/ LR	SAG (R)	5.63 ± 0.57
17.	K132	F/24	Munger/ LR	ND	3.95 ± 0.28
18.	K133	M/20	West Bengal/LR	SAG (R)	3.45 ± 0.28
19.	K135	F/45	Gopalganj/LR	SAG (R)	4.22 ± 0.38
20.	K216	M/14	West Bengal/LR	SAG (R)	2.14 ± 0.28

[a]HR= high resistance, LR = low resistance zone.

[b]R= responsive to SAG or AmB, NR= not responsive, NA = not applicable. Response to treatment was noted after 30 days treatment with SAG 20mg/kg body wt. and with *AmB* infusions of 1 mg/kg on alternate days for 1 month. Patients with absence of fever and with reduction in spleen size were designated as responsive (R) while others were considered non-responsive (NR).

[c]Mean ED_{50}±SD from 3 separate assays

[d]Patient died during the treatment, ND= Not determined.

Comparison of ED_{50} values of isolates from different endemicity zones and correlation with SAG susceptibility

The mean/ median ED_{50} values at the clinically relevant stage i.e. intracellular amastigote were compared in isolates originating from high and low endemicity zones which show different susceptibility to SAG as defined by Sundar et al., [2001b]. The mean ED_{50} of isolates from HR region (15.81 ± 2.50 μg/ml) was significantly higher ($p<0.001$) in comparison to the ED_{50} of LR (5.46 ±3.69 μg/ml) region isolates for SAG. The median ED_{50} from HR region was 15.76 μg/ml and LR region was 4.22 μg/ml respectively. We observed a strong correlation of *in vitro* SAG susceptibility with the endemicity zones (r_{rank}= 0.998) of the isolates and with the clinical response for SAG (r_{rank}=0.982).

Comparison of gene expression profiles between sensitive and resistant *L. donovani*

Gene expression studies were performed between SAG sensitive K135 (ED_{50} = 4.22 ± 0.38 μg/ml) and SAG resistant K80 SbIII (ED_{50} = >100 μg/ml). Changes in mRNA abundance during differentiation of sensitive vs resistant were examined by genome-wide expression profiling using genomic DNA microarrays. The hybridized scanned array was subjected to analysis as described in Materials and Methods (Fig 4.2). Multiple replicates of all hybridizations were performed to account for sample heterogeneity and possible variation due to hybridization. A series of replicate experiments using the same RNA sample were done initially to obtain an estimate of the accuracy and precision of the system. Microarrays were hybridized with fluorescently labeled Cy3 and Cy5 cDNA, both prepared from promastigote RNA. This

theoretically should give a log expression ratio (Cy5/Cy3) of 1 for all the elements arrayed on to the slide.

Replicate experiments with three biological preparations were performed comparing sensitive and resistant parasites. A representative image of a hybridized microarray comparing sensitive and resistant is shown in Fig. 4.1. Panel A shows the image of a hybridized array captured with laser scanner. Panel B and C represents enlarged view of the blocks from the array. The array quality control report was generated before going for further analysis; various criteria for quality control as defined by the software (GenePix Pro 5.0) were passed.

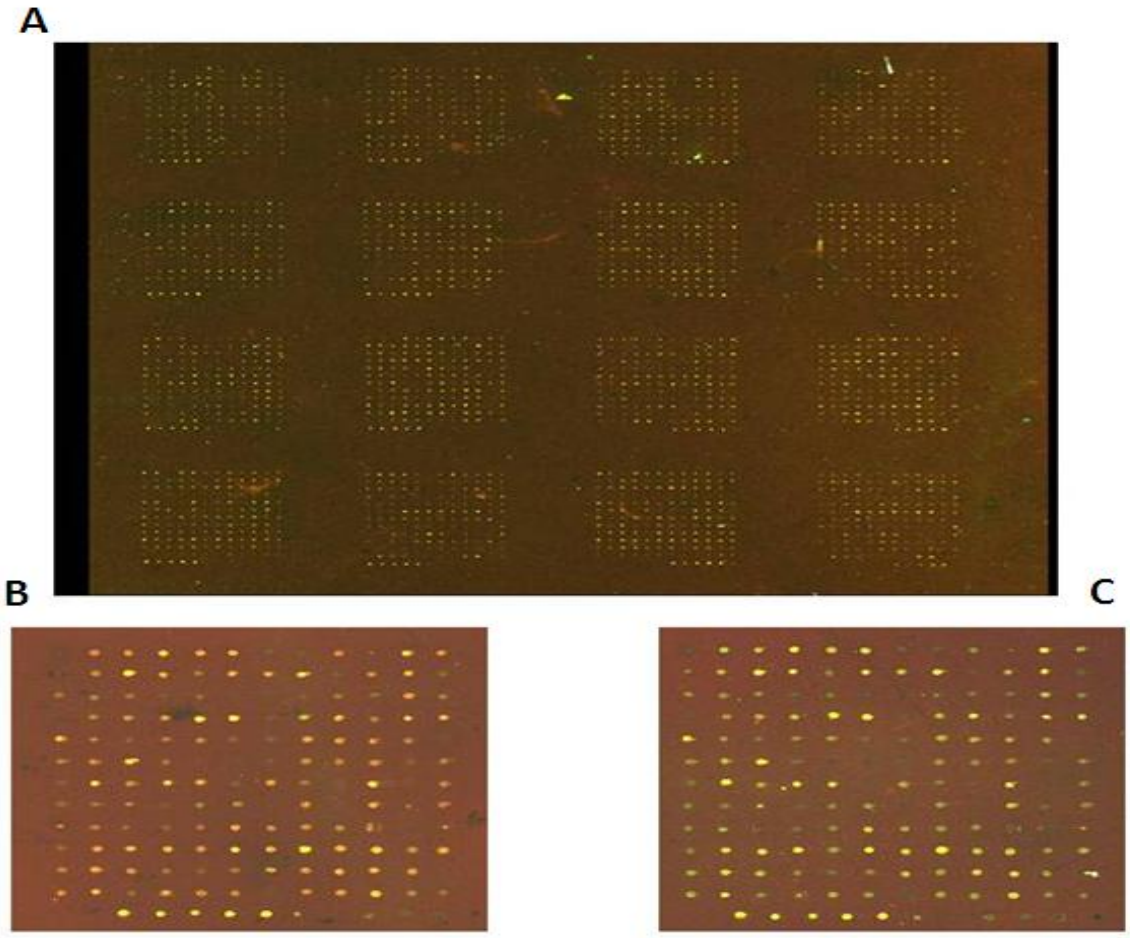


Fig 4.1 Microarray image using sensitive and resistant RNA samples. Panel A shows the image of a portion of microarray with 16 blocks. Panel B and C show image of a single block from two dye-swap hybridizations, image captured with laser scanner. Panel B shows resistant labeled with Cy5 and sensitive with Cy3, panel C shows the reverse. Spike-in controls are in the row at the bottom of each panel, position 1 to 5 from the left. The remaining spots in the control row are negative controls: 3XSSC, Salmon DNA, tRNA, poly Adenosine, human cot-1 DNA from left to right. Total RNA was used to synthesize Cy3 or Cy5 labeled cDNA and hybridized to the DNA chip under stringent conditions.

To adjust for unequal fluorescence intensities of the two RNA samples and to allow comparison from experiment to experiment, the data was normalized using Acuity 3.1 software. Normalization was carried out based on the premise that most genes on the array are not differentially expressed; therefore, the arithmetic mean of the ratios from every feature on the array is equal to 1. To produce a continuous distribution of up and down regulated spots, the ratios were transformed to the $\log_2$ scale. Further normalization to account for the systematic dependence of ratio on intensity was performed by locally weighted linear regression (LOWESS). Scatter plot of a representative hybridization comparing the expression in resistant vs sensitive following LOWESS normalization is shown in Fig. 4.2. From the scatter plot, it is seen that the majority of the log ratio values are clustered close to zero reflecting no change in expression as expected, with outliers representing the differentially expressed genes. Similar plots were obtained in all the hybridizations comparing sensitive and resistant RNA expression (Data not shown).

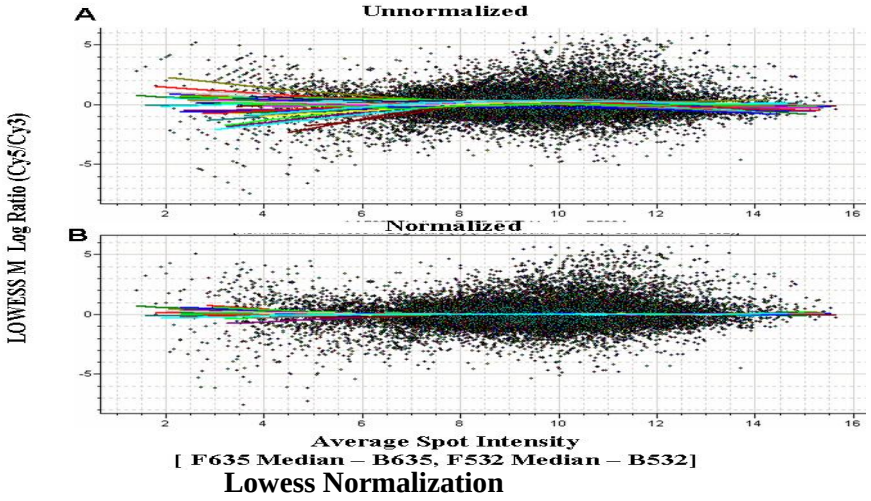


Fig. 4.2 Panel A shows scatter plot comparing the $\log_2$ ratios of fluorescence intensities of each spot against the product of intensities before normalization. Panel B shows the same following LOWESS normalization using Acuity software.

Expression profiling of drug-resistant genes

Analysis of microarray experiments revealed a number of DNA clones showing differential expression in drug resistant and sensitive promastigotes. Robust statistical methods were then applied for these differentially expressed genes. Z scores for each gene were computed as the ratio of mean difference between the two groups for each gene, divided by standard error for the corresponding gene. Z score values are used as the data basis in calculations of Z-ratio. Of these, the clones showing significant and consistently higher expression with ratio ≥ 1.4, in four microarray hybridizations and reproducibility in dye flip microarrays experiments were chosen. The higher the Z score, the greater is the confidence that the transcript is differentially expressed between the 2 phenotypes. The clones meeting these criteria were selected and sequenced using single pass automated sequencer for further analysis. The clone sequences were searched by BLAST in the *L. infantum* genome for their gene identity.

A total of 11 clones out of 8448 were identified to be over-expressed in promastigotes of the resistant isolate K80SbIII and 1 gene was over expressed in the sensitive isolate K135 (Table 4.3). From the selected DNA clones, 5 clones 68F10, 28F11, 90A5, 69E3 and 78B4, showed homology with MAP-kinase, two other clones, 51E7 and 62D4, were homologous to Histone H4, one clone 87A9 was homologous to Histone 2A, one clone, 47F10, showed homology to Histone H1 and two Clones 58A8 and 87B9 showed homology to uncharacterized conserved hypothetical protein coding genes. From the clones down regulated in resistant parasites, 42G8 showed homology to an amino acid transporter.

Table 4.3: Differentially expressed genes in resistant vs. sensitive parasites as determined by microarray and RT-PCR analysis.

Clone ID	**Gene**	**Gene DB** **Systematic ID**	**Res/Sens** **Median of ratios± SD**	**Z-Ratio**	**P-value**	**RT-PCR** **Fold change**
47F10	Histone H1	LinJ27_V3.1120	1.53 ± 0.22	3.55	0.001	1.52
87A9	Histone 2 A	LinJ29_V3.1860	1.78 ± 0.53	3.84	0.001	3.00
62D4	Histone H4	LinJ36_V3.0020	2.14 ± 0.50	3.14	0.001	2.50
51E7	Histone H4	LinJ36_V3.0020	1.79 ± 0.43	3.16	0.001	2.50
68F10	MAP Kinase	LinJ36_V3.6760	1.70 ± 0.42	3.92	0.001	3.50
28F11	MAP Kinase	LinJ36_V3.6760	1.64±0.26	3.72	0.001	3.50
90A5	MAP Kinase	LinJ36_V3.6760	1.62 ± 0.45	2.16	0.003	3.50
69E3	MAP Kinase	LinJ36_V3.6760	1.52 ± 0.12	3.75	0.001	3.50
78B4	MAP Kinase	LinJ36_V3.6760	1.42± 0.22	2.44	0.002	3.50
42G8	Amino Acid transporter	LinJ31_V3.0350	0.61± 0.13	2.56	0.002	0.46
87B9	Hypothetical protein	LinJ35_V3.3990 73.1 kDa	1.40 ± 0.26	1.49	0.01	2.00
58A8	Hypothetical protein	LinJ04_V3.0630 23.3 kDa	1.70 ± 0.36	3.37	0.001	7.10

3.3 Validation of microarray results by RT-PCR and western blotting:

The differential expression observed in microarray analysis was evident in RT-PCR analysis (Fig. 4.3). The fold difference of the gene expression from the microarray as well as RT-PCR analysis is given in Table 4.3 and the data is consistent in both the analysis.

The expression changes were further verified at the protein level by western blotting with the one available antibody to us, H2A. Expression at the protein level matched with the expression at the RNA level, being 2.5 fold higher in resistant parasites in comparison to sensitive (Fig. 4.4). The higher expression of the molecule at the transcript as well as protein level makes it an attractive candidate as drug resistance biomarkers to be tested in field isolates.

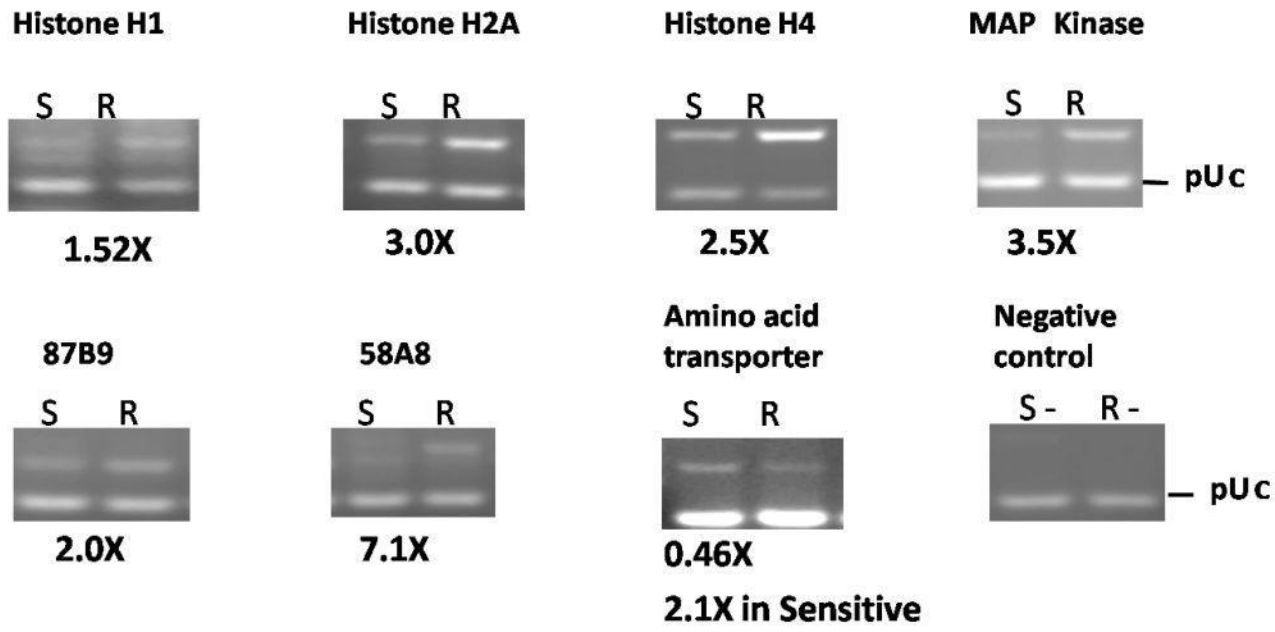


Fig. 4.3. RT-PCR analysis of differentially expressed genes in antimony sensitive K135 (S) and resistant K80 SbIII(R) *Leishmania* parasites. In all the panels, the lower band (100bp-pUC) represents the amplification of pUC plasmid sequence spiked in the cDNA preparation from resistant or sensitive parasite, while the upper bands represent the amplification of the gene sequence written below the panel. Negative control experiments were performed with amplification using total RNA but no reverse transcriptase to check for DNA contamination. Expression fold change in resistance with respect to sensitive is shown in the term of X -fold.

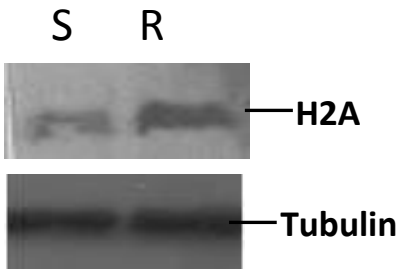


Fig. 4.4. Validation of microarray results by Western blotting. Expression pattern was verified in the total promastigote lysate of the parasite used in the microarray. Protein lysates (100µg) from K80 SbIII (R) and K135 (S) were electrophoresed and probed with Histone H2A primary antibody at a dilution of 1:500 and subsequently with secondary antibody rabbit IgG conjugated with HRP (1:10,000). The blots were developed using western blot detection enhanced chemiluminescent (ECL) detection reagent. The results shown are from a single experiment typical of at least three giving identical results. The blot was rebound with an α-tubulin antibody to monitor the amount of protein lysates loaded on the gel. Lane 1- sensitive isolate and Lane 2- resistant isolate.

Discussion:

SAG has been the first drug of choice against Leishmaniasis and resistance to this drug is a major problem in the field not only in the Indian subcontinent, but also throughout the world (Lira et al., 1999; Singh et al., 2006; Hadighi et al., 2006; Rojas et al., 2006). The increase in resistance to SAG has led to an upsurge in therapeutic failure, and with the limited chemotherapeutic alternatives, understanding the mechanisms responsible for the resistance could help lead to effective drug treatment strategies.

DNA microarrays either in the form of random genomic fragments or cDNA have proved their utility in several studies dealing with assessment of *Leishmania* gene expression during parasite differentiation on a genome-wide scale that allowed chronicling of the molecular events during stage transition (Srividya et al., 2007), and differences between KA and PKDL (Salotra et al., 2006). The present study considered 19 *L.donovani* isolates from Indian patients of VL who were from zones of varying degree of endemicity for VL. *In vitro* susceptibility of parasites isolated from SAG treated (responsive and non responsive) and AmB treated patients (all responded to treatment and no clinical resistance was observed) was studied. The field isolates of *L. donovani* were investigated for susceptibility towards SAG using *in vitro* assay at intracellular amastigote stage, further DNA microarray technology was employed to characterize alterations in gene expression that occur in SAG resistant and sensitive *L.donovani*. The genomic shotgun clones that were arrayed on the microarray used in this study represent an unbiased sample of the *L. donovani* genome.

Results indicated a strong correlation between the isolates from different geographical regions of Bihar and *in vitro* susceptibility of intracellular amastigotes, also the isolates from HR

region had lower susceptibility than those from LR region isolates. The drug susceptibility of patients isolates showed a good correlation with the clinical response of the patients to SAG.

K80 and K135 were isolated from clinically confirmed SAG responsive and unresponsive patients respectively (Table 4.2). The drug susceptibility of these isolates was consistent even after repeated passages in cultures as promastigotes and amastigotes in an intracellular assay recapitulating the parasitic behavior in patients. Further K80 was adapted as SbIII resistant parasite by *in vitro* passages with a stepwise increase in the concentration of SbIII from 10 to 125 µg/ml(K80SbIII) in the medium M199 + 10 % FBS.

Having established parasite resistance to drug by observing the relationship between SAG sensitivity of K135 and K80SbIII *in vitro*, we pursued transcriptome analysis on these isolates. Using the rigorous statistical methods rather than simple fold changes for analysis of DNA microarray experiments we identified 11 clones which were up-regulated in the resistant isolate and one clone (amino acid transporter) in the sensitive isolate. Blast analysis using *L. infantum* Gene DB revealed that out of the 11 up-regulated genes in the resistant isolate, 4 belong to the Histone family (Histone H1, H2A and H4) and 5 were various segments of the MAP Kinase-1 gene, while two of them were hypothetical proteins with unassigned functions. Further, the differential expression of these genes in sensitive and resistant parasites was validated by RT-PCR analysis. Expression patterns of H2A gene was validated at the protein level.

Our observation of very few genes with modulated expression in the resistant parasite is consistent with other studies on drug resistant parasites where by whole genome expression profiling revealed only 24 genes as differentially expressed in antimony resistant parasites. In

methotrexate resistant parasites again very few genes were differentially expressed without concomitant change in copy number. In both the studies, modulation in gene expression was associated with gene amplification and gene deletion (Ubeda et al., 2008; Lephrohon et al., 2009).However, in resistant field isolates, researchers often failed to detect gene amplification events indicating that alteration in RNA expression or the point mutations may be responsible for the resistant phenotype (Singh et al., 2003; Singh et al., 2006; Rojas et al., 2006,).

Mitogen-activated protein (MAP) kinases are well-known mediators of signal transduction of higher eukaryotes regulating important processes like proliferation, differentiation, cell shape, stress response and apoptosis. Of the 17 Mitogen-activated protein (MAP) kinases and MAPK-like kinases identified in *Leishmania* (Wiese 1998; Kuhn et al., 2005; Parsons et al., 2005), we observed consistent upregulation of MAPK1 in the resistant isolate. MAP Kinases play a significant role in parasite intracellular proliferation, flagellar morphogenesis and hence parasite virulence during mammalian infection (Wiese 1998; Bengs et al., 2005; Wang et al., 2005; Erdman et al., 2006). Genetic studies using *Leishmania mexicana* MAPK null mutant parasites (LmxMPK) revealed that inactivation of LmxMPK1 abrogated parasite virulence during mammalian infection due to a defect in intracellular proliferation, and null mutants of LmxMPK3 and LmxMPK9 showed defects in flagellar morphogenesis, that may have important consequences for sandfly infection and parasite transmission (Wiese 1998; Bengs et al., 2005; Erdman et al., 2006). We found 5 up-regulated DNA clones mapping to MAP Kinase-1 in resistant isolate, emphasizing its role in drug resistance. The exact mechanism how MAPK1 contributes to antimony resistance remains to be explored.

Leishmania parasites possess core histones H2A, H2B, H3, H4 (Soto et al., 1992, 1997) and linker histone H1 (Fasel et al., 1993; Martinez et al., 2002; Papageorgiou & Soteriadou 2002) that facilitate the formation of higher order chromatin structures. In eukaryotes, posttranslational modifications of core histones act in diverse biological processes such as gene regulation, DNA repair and chromosome condensation (mitosis). Histone synthesis in *Leishmania* is tightly coupled to DNA replication by a post-transcriptional mechanism operating at the level of translation (Abanades et al., 2009). Very few studies have been carried out on trypanosomatid histone modifications and on their role in gene regulation. Recently, a CHIP-Chip assay revealed that H3 histones at the origins of polycistronic transcription of protein-coding genes are acetylated and possible modification in the acetylation state of these origins regulates transcription initiation (Thomas et al., 2009). However, the role of histone proteins in antimony resistance has not been established, in the present study of the 11 DNA clones up-regulated in resistant parasites four were corresponding to histones namely H1, H2A and H4. In *Leishmania,* histone mRNA levels are regulated by a mechanism coupled to cellular growth. During promastigote growth, histone H1 mRNA progressively accumulates from early log phase to stationary phase. Addition of histone H1 affects chromatin condensation of parasite nuclei and its over-expression in *Leishmania* has resulted in the reduced infectivity of the parasite *in vitro* as well as *in vivo* (Smirilis et al., 2006; Masina et al., 2007). We observed 1.5 fold upregulation of histone H1 at the RNA level in a resistant isolate .

We observed up to 2 fold changes in expression of core histones H2A and H4 by microarray and similar results were obtained by RT-PCR. The expression of H2A was found to be elevated at the protein level signifying its functional role. Unlike histone H1, decrease in

RNA levels, associated with the growth phase, has been observed for other *L. Infantum* histones, H2A, H3 and H4 (Soto et al., 1992,1996,1997). We used the identical growth points for comparative expression profiling eliminating the possibility of growth related differential expression of histones.

Our array identified several differentially expressed molecules that might enable the parasite to survive within the phagolysosome of vertebrate macrophages and its transmittance by sand fly vectors. The functional study of the identified genes will provide useful insights into their role in development of resistance in *Leishmania*. Such genes may have potential as biomarkers of drug resistance in VL.

Introduction:

The first line treatment for VL is a pentavalent antimony (SbV) sodium antimony gluconate (SAG) as recommended by the World Health Organization (WHO, 1990), despite its requirement for long courses and cardiac toxicity. In India, as many as 65% of previously untreated patients fail to respond promptly or relapse after therapy with antimony drugs, due to the development of drug resistance (Sundar, 2001a).

Earlier studies using isolates from responsive and non responsive patients indicated that resistance to antimonials is an intrinsic parasite property (Lira et al.,1999). Development of drug resistance appears to be a feature of intensive transmission of *L. donovani* in Bihar possibly due to anthroponotic transmission (Singh et al., 2006). Antimony resistant *Leishmania* parasites have been observed in several endemic regions like Iran, Peru, Columbia (Hadighi et al., 2006; Rojas et al., 2006; Yardley et al., 2006). The resistance to antimonials varies with the degree of disease endemicity and in our previous study we demonstrated that the mean ED_{50} of the isolates from high endemicity zone was significantly higher than the mean ED_{50} of isolates from low endemicity zone, emphasizing the acquired resistance in the region (Singh et al., 2006). In the context of the VL elimination initiative need is expressed for validated assays for the surveillance of drug resistance *Leishmania* parasite (Boelart, 2007). A field based assay capable of distingushing drug sensitive/resistant parasite will facilitate the control of the disease.

Determinants of resistance in laboratory strains are partly known, however the mechanism operating in field isolates is not well understood. Data from *in vitro* selected antimony resistant strains revealed that antimony resistance is multifactorial and one or more

simultaneously present mechanisms including loss in metal reduction, over-expression of thiol biosynthetic enzymes and the SbIII/thiol conjugate sequestering pump, and the decreased Sb(III) uptake due to lower level of expression of the gene Aquaporin-1 (AQP1) (Ouellette, 2004; Croft et al., 2006) may be responsible for the phenotype. This does not exclude that the parasite could have other mechanisms that confer metal resistance. In recent times using various methods like gene expression profiling, comparative proteomic analysis and functional cloning, several other genes and gene products implicated in antimony resistance have been identified. These include heat shock protein 83 (HSP 83) (Vergenes et al., 2007), a high molecular weight protein P228 (Choudhury et al., 2008), argininosuccinate synthetase (ARGG), kinetoplastid membrane protein (KMP-11) (El Fadili et al., 2009), and a leucine rich repeat (LRR) superfamily protein (Genest et al., 2008). Our studies on transcriptome profiling of antimony resistant field isolates led to identification of many new candidates including parasite surface antigen 2 (PSA-2), nucleoside transporter, Histones and MAP Kinase1 (Salotra et al., 2005; Chapter-4).

These observations prompted us to examine the expression of various genes putatively implicated in antimony resistance (MRPA, GSH, AQP1, HSP83, PSA-2 reported in earlier studies and MAP Kinase, Histone H1, H2A and H4 identified in chapter-4) in natural antimony resistant and sensitive *L. donovani* strains isolated from VL patients in order to understand the mechanism of resistance and identify potential biomarkers of drug resistance.

Materials and Methods

Parasite and culture conditions

Promastigotes of *L. donovani* strains *Ld*AG83 (MHOM/IN/80/AG83), a lab generated SAG resistant isolate K80SbIII along with 14 *L.donovani* field isolates (4 sensitive and 10 resistant) described in chapter-4 have been employed in this study. Isolates from VL patients were designated as SAG sensitive (S) and SAG resistance (R) based on their response to treatment and ED_{50} value at amastigote stage, as described in chapter-4.

RNA isolation, cDNA synthesis and Real-time quantitative PCR.

Promastigotes were cultured and harvested at mid log phase and immediately disrupted in Trizol reagent for RNA extraction following instructions recommended by the manufacturer. All samples were treated using the DNAse I to remove possible contaminating genomic DNA.

RNA and cDNAs were prepared as described in chapter-4.The resulting cDNA was diluted 10 times, and 2 µl was added to 25 µl quantitative PCRs for expression analysis of nine genes coding for proteins putatively involved in Sb (V) metabolism, referred to as target genes, and of Glyceraldehyde phospho dehydrogenase (GAPDH) gene included for normalization purpose, referred to as internal control. We chose GAPDH gene for normalization process as this gene is expected to have a stable expression. The sequences of the primers for all the genes amplified in the study are given in the Table - 5.1.

All real time PCR reactions were performed in triplicate in 25µl volumes using SYBR Green PCR master mix for detection in an ABI Prism 7000 Sequence Detection System (Applied

Biosystem). A singleplex reaction mix was prepared according to the manufacturer's protocol. The reactions were run using the thermal profile consisting of three stages: (1) incubation at 50ºC for 2 min, (2) incubation at 95ºC for 10 min, and (3) incubation at 95ºC for 15 sec, then at 60ºC for 1 min. The third stage was repeated for 40 cycles. A dissociation protocol was performed with 60°C set as the starting temperature with increments of 0.5°C every 30 s to ascertain amplification of the expected product and to ensure no nonspecific products or primer dimers (which could bias the quantification) were formed. The negative controls of cDNA synthesis (i.e., without reverse transcriptase), and no-template controls were included in each run for each gene. All reactions were done in triplicate and the arithmetic average threshold cycle (Ct) was used for data analysis.

Analysis of gene expression using the $2^{-\Delta\Delta Ct}$ method.

We used the $2^{-\Delta\Delta Ct}$ method to calculate relative changes in gene expression determined from real time quantitative PCR experiments as described earlier (ABI, 1997). The data are presented as the fold change in the target gene expression in the *L. donovani* field isolates normalized to the internal control gene (GAPDH) and relative to the reference isolate of *L. donovani Ld*AG83. Results of the real time PCR data were represented as Ct values. The average Ct was calculated for both the target genes and GAPDH and the ΔCt was determined as (Ct values for the target gene) minus (Ct values for GAPDH). The -ΔΔCt represents the difference between the paired isolate samples, as calculated by the formula -ΔΔCt = (ΔCt of field isolate – ΔCt of *Ld*AG83). The N-fold differential expression in the target gene of a field isolate compared to the *Ld*AG83 sample counterpart was expressed as $2^{-\Delta\Delta Ct}$. In the present study, mRNA

expression profile categorized as follows: N fold≥ 1.5, designated as increased, N-fold ranging from -1.49 to 1.49 as comparable, and N-fold ≤ -1.5 as decreased.

Western blot analysis

The method of western blotting has been described in chapter-4. Monoclonal PSA-2 antibody was a kind gift from Dr. Handman (Walter Eliza Health Institute, Melbourne, Australia.) Antibody to H2A was obtained as a kind gift from Dr. Jose M Requena (Universidad Auto!noma de Madrid, Spain).

Table 5.1: Chosen target genes their functions and primers used for amplification.

S. N.	GENE	Protein/ GENE ID (*L.infantum* databse)	Function/ Relevance	Forward and Reverse Primers (5'-3')	Product size (bp)
1.	MRPA	Multidrug resistance protein A/ LinJ23_V3.0290	Sequestration of Sb(III) thiol conjugate, over expression linked to Sb(III) resistance	F-GCGCAGCCGTTT GTG CTT GTG G R-TTG CG TAC GTC GCG ATG GTG C	179
2	GSH	gamma-Glutamylcysteine synthetase/ LinJ18_V3.1660	Thiol biosynthesis; overexpression linked to Sb(III) resistance	F-CATTGG CTG GCG CGT TGA GTT C R-ATG TGC GCG GCC CAT ATT CTC G	166
3.	AQP-1	Aquaglycerporin-1/ LinJ31_V3.0030	Uptake of SbIII	F-TTT GGA ACC GGC GTC GTT GC R- ACA CAG TTC GCC AGC GTT ACG G	182

4.	HSP83	Heat Shock Protein 83/ LinJ33_V3.0350	Role in antimonial resistance by modulating drug-induced programmed cell death.	F-GCG CAA CAA CAT CAA GCT GTA CG R-GTT CTT GCG GAT CAC CTT CAG GA	175
5	PSA-2	Parasite Surface Antigen-2/ LinJ12_V4.0671	membrane proteins involved in parasite-to-host physical interactions	F-CGT GCG ATC CCT GAG CTT R-CCG GCA TAC TTT GGC TGA AA	106
6.	MAPK-1	Mitogen Activated Protein Kinase/ LinJ36_V3.6760	In signal transduction regulating proliferation, differentiation, stress response and apoptosis	F-GGT GTG TGA TTG GGG AGA TGC R- TCG CCT CGC TAT CCT TCA GG	219
7.	H1	HistoneH1/ LinJ27_V3.1120	Linker Histone of Nucleosome	F- CTC GCC GCA GAA GTC TCC R- TTC TTC GCC GAG GAT TTC	238
8.	H2A	Histone2A/ LinJ29_V3.1860	Core histone protein of Nucleosome	F-CTG CCG TGC TGG AGT ACC TG R-TGT CGC CTT GCC CTT CTT G	233
9.	H4	HistoneH4/ LinJ36_V3.0020	Core histone protein of Nucleosome	F- ATG GCC AAG GGC AAG CGC CT R-CGC CGT CAC CGT CTT CTTG	246

10	GAPDH	Glyceraldehydes-3-phosphate dehydrogenase/ LinJ30_V3.2990	Internal control	F-GAA GTA CAC GGT GGA GGC TG R-CGC TGA TCA CGA CCT TCT TC	206

Results:

In order to understand the mechanism of antimony resistance operative in the field, the expression of 9 genes was evaluated in 14 *L.donovani* field isolates, including 4 sensitive (ED_{50} range, 2.14 ± 0.28 to 5.63 ± 0.57) and 10 resistant isolates (ED_{50} range, 11.82 ± 1.28 to 20.30 ± 0.84), as reported in chapter-4, in comparison with *Ld*AG83, a standard sensitive *L. donovani* isolate.

Characterization of *in vitro* induced antimony resistant strain K80 SbIII

Initially we analysed the expression of all 9 genes in *in vitro* induced antimony resistant *Leishmania* strain K80 SbIII depicted in Fig 5.1.The ED_{50} of K80SbIII for SAG was >100 µg/ml while for *Ld*AG83 it was 2.06 ± 0.23µg/ml (Chapter-4). The expression pattern of various genes was in agreement with previous reports i.e. the expression of 8 genes was several fold high in comparison to the sensitive strain *Ld*AG83 while expression of AQP1 was down regulated in the *in vitro* induced resistant strain (Fig 5.1a). Further, the expression of H2A was 1.5 fold high while PSA-2 was 2.5 fold high in K80 SbIII wrt *Ld*AG83 at protein level (Fig 5.1b, c).

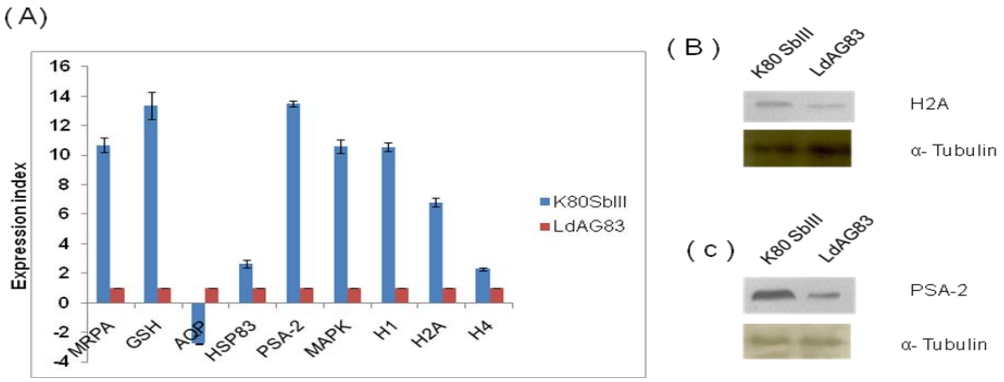


Fig 5.1. Characterization of *in vitro* induced antimony resistant strain K80 SbIII. (A) Expression pattern of various genes in resistant *Leishmania* strain analyzed using Real Time PCR and represented as expression index with respect to *Ld*AG83. Values given are mean ± SD of three different experiments. (B) Expression of H2A protein and (C) PSA-2 protein.

Expression analysis of Target genes in Indian field Isolates of Kala-azar:

The gene expression analyses of the target genes in 4 sensitive and 10 resistant field isolates with respect to *Ld*AG83 are given (Table 5.2 a,b & Figure5.2 a,b). Expression of MRPA was ≥1.5 fold high in 9/10 resistant parasites and ranged up to 10.7 fold in comparison to *Ld*AG83, however it was comparable to *Ld*AG83 in 1 resistant isolates. On the other hand, high expression of MRPA was observed in one sensitive field isolate. The correlation coefficient between expression of MRPA and ED_{50} of the isolates was 0.45. Expression of GSH was low in all sensitive isolates and it was 1.9 to 13.4 fold high in 8 of 10 resistant isolates. A moderate positive correlation (r= 0.60) was observed between expression of GSH and ED_{50} of the isolates. Expression of Sb (III) transport protein AQP1 was up-regulated in all the sensitive isolates by 2.6 fold to 7.5 fold indicating the increased uptake of antimony and thereby the higher drug susceptibility of these isolates. In resistant isolates the gene expression was marginally less or

comparable to *Ld*AG83 in 6 of 10 while in other 4 it was significantly less (1.8 to 6.2 fold). We observed a moderate negative correlation (r= -0.60) of the expression of AQP1 with the ED_{50} of the isolates.

Expression analysis of recently identified target molecules revealed interesting results in field isolates. Expression of HSP83, PSA-2 and the three histones H1, H2A and H4was down regulated or comparable to *Ld*AG83 in all 4 sensitive isolates while MAPK1 showed high expression in one sensitive isolate. We observed ≥1.5 fold high expression of PSA-2 and MAPK1 in 10/10, H1 in 9/10, H2A in 8/10, H4 in 7/10 and HSP83 in 6/10 resistant isolates with respect to *Ld*AG83. We observed a strong positive correlation between gene expression and ED_{50} of the isolates for PSA-2 (r= 0.79) and H2A (r= 0.83), while the correlation was moderate for H4(r= 0.67) and HSP83 (r= 0.63) and low for H1(r= 0.51) and MAPK1 (r = 0.26). Further the two genes H2A and PSA-2 showing strong correlation of expression with ED_{50} were analysed at protein level by western blotting in parasite lysates. The expression of both PSA-2 and H2A was high in the resistant isolates as compared with the sensitive isolates (Figure 5.3 a, b).

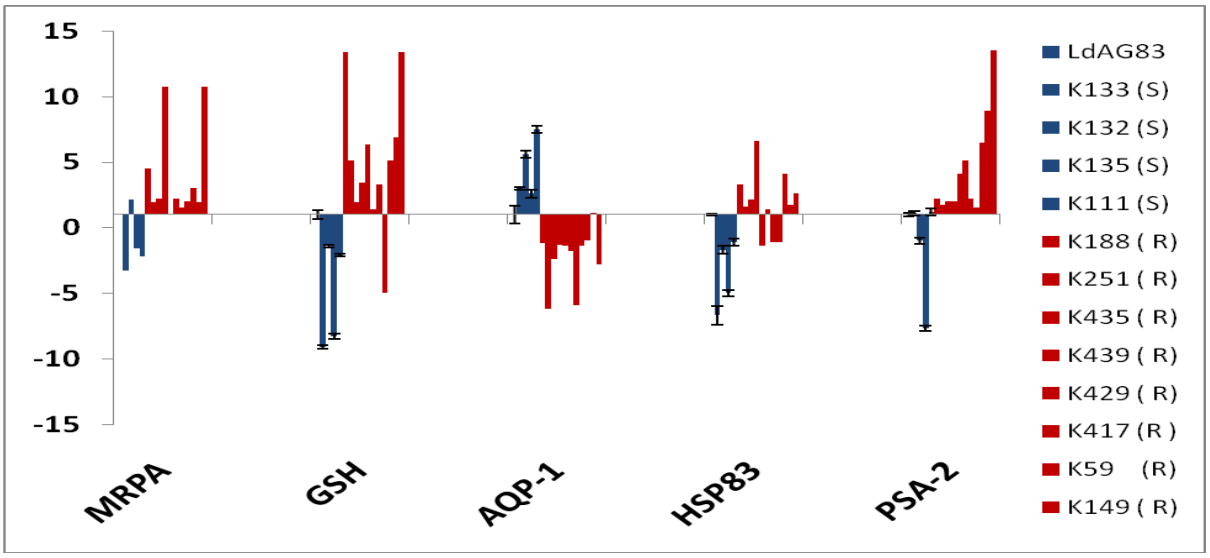
Table 5. 2 (A) -: Expression level of different genes implicated in antimony resistance in Indian field isolates, with respect to *Ld*AG83

S.N	Parasite ID	SAG ED_{50} (µg/ml)	MRPA	GSH	AQP-1	HSP83	PSA-2
1	*Ld*AG83	2.06 ± 0.23	1	1	1	1	1
2	K133 (S)	3.45 ± 0.2	-3.3±0.25	-9.1±0.12	3.0 ±0.11	-6.7±0.09	1.1±0.11
3	K132 (S)	3.95 ± 0.2	2.1±0.12	-1.4 ±0.12	5.6±0.32	-1.7±0.21	-1.0 ±0.11
4	K135 (S)	4.22 ± 0.3	-1.6±0.11	-8.3±0.21	2.6±0.16	-5.0±0.31	-7.7±0.17
5	K111 (S)	5.63 ± 0.5	-2.2 ±0.24	-2.1± 0.11	7.5 ± 0.69	-1.1± 0.31	1.2 ± 0.27
6	K188 (R)	11.27 ± 0.8	4.5±0.98	13.4 ± 0.92	-1.2 ± 0.17	3.3±0.22	2.2±0.21
7	K251 (R)	11.82± 1.2	1.9±0.31	5.1 ± 0.72	-6.2 ± 0.09	1.6±0.25	1.7±0.29
8	K435(R)	11.82± 1.3	2.2±0.25	1.9±0.34	-2.4±0.19	2.1±0.14	2.0±0.21
9	K439 (R)	12.88± 0.1	10.7 ±0.23	3.4 ±0.19	-1.3±0.12	6.6±0.21	2.0±0.26
10	K429 (R)	13.76± 0.8	1.0±0.04	6.3± 0.25	-1.4 ±0.09	-1.4±0.11	4.1±0.56
11	K417 (R)	14.65± 0.6	2.2±0.26	1.4±0.36	-1.8 ± 0.11	1.4±0.23	5.1±0.61
12	K59 (R)	14.66± 3.2	1.5±0.32	3.3±0.34	-5.9±0.07	-1.1±0.11	2.2±0.45
13	K149 (R)	15.7± 4.0	2.0±0.34	-5.0 ± 0.23	-1.4 ± 0.09	-1.1±0.13	1.5±0.14
14	K131 (R)	19.38± 1.6	3.0±0.62	5.1 ±0.87	1.0 ± 0.11	4.1±0.54	6.5±0.92
15	K192 (R)	20.3± 0.8	1.9 ± 0.2	6.9 ± 0.56	1.1 ± 0.12	1.7±0.24	8.9±0.76
16	K80SbIII (R)	>100	10.7±0.5	13.4±0.9	-2.8±0.01	2.6 ±0.26	13.5±0.2

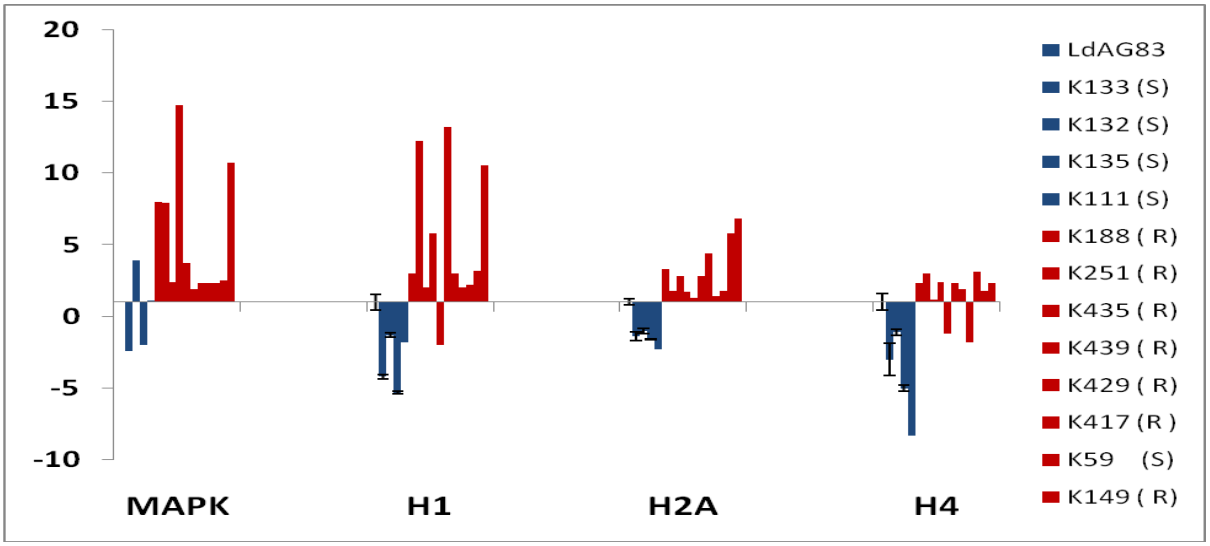
R: Resistant, S: Sensitive

Table 5.2 (B) -: Expression level of different genes implicated in antimony resistance in Indian field isolates, with respect to *Ld*AG83

S.N	Parasite ID	SAG ED_{50} (µg/ml)	MAPK	H1	H2A	H4
1	*Ld*AG83	2.06 ± 0.2	1	1	1	1
2	K133 (S)	3.45 ± 0.2	-2.4±0.11	-4.2± 0.14	-1.4±0.14	-3.0±0.22
3	K132 (S)	3.95 ± 0.2	3.9±0.54	-1.3± 0.15	-1.0±0.14	-1.1±0.09
4	K135 (S)	4.22 ± 0.3	-2.0±0.22	-5.3±0.26	-1.6±0.21	-5.0±0.33
5	K111 (S)	5.63 ± 0.5	1.1 ± 0.21	-1.8 ±0.29	-2.3± 0.19	-8.3 ± 0.03
6	K188 (R)	11.27 ± 0.8	8.0 ± 0.45	3.0± 0.35	3.3± 0.43	2.3±0.14
7	K251 (R)	11.82± 1.2	7.9±0.59	12.2± 1.1	1.8±0.22	3.0±0.21
8	K435(R)	11.82± 1.3	2.4±0.19	2.0 ± 0.12	2.8±0.16	1.2±0.12
9	K439 (R)	12.88± 0.1	14.7±0.62	5.8±0.34	1.7 ±0.15	2.4±0.32
10	K429 (R)	13.76± 0.8	3.7±0.12	-2.0±0.07	1.3 ±0.09	-1.2±0.11
11	K417 (R)	14.65± 0.6	1.9±0.11	13.2±1.8	2.8±0.12	2.3±0.23
12	K59 (R)	14.66± 3.2	2.3±.36	3.0±0.89	4.4±0.48	1.9±0.19
13	K149 (R)	15.7± 4.0	2.3±0.14	2.0 ±0.11	1.4±0.11	-1.8±0.12
14	K131 (R)	19.38± 1.6	2.3 ±0.41	2.2±0.13	1.8 ± .21	3.1±0.12
15	K192 (R)	20.3± 0.8	2.5±0.24	3.2±0.12	5.8±1.2	1.8±0.19
16	K80SbIII (R)	>100	10.7±0.45	10.5±0.31	6.8 ± 0.29	2.3±0.11



(A)



(B)

Figure 5.2: Expression pattern of various drug resistance associated genes in *L. donovani* field isolates. Gene expression was analysed using Real Time PCR and represented as expression index with respect to *Ld*AG83. Values given are mean ± SD of three different experiments. (A): Expression of MRPA, GSH1, AQP1, HSP83 and PSA-2. (B): Expression of MAPK-1, Histones H1, H2A and H4 in *L. donovani* field isolates.

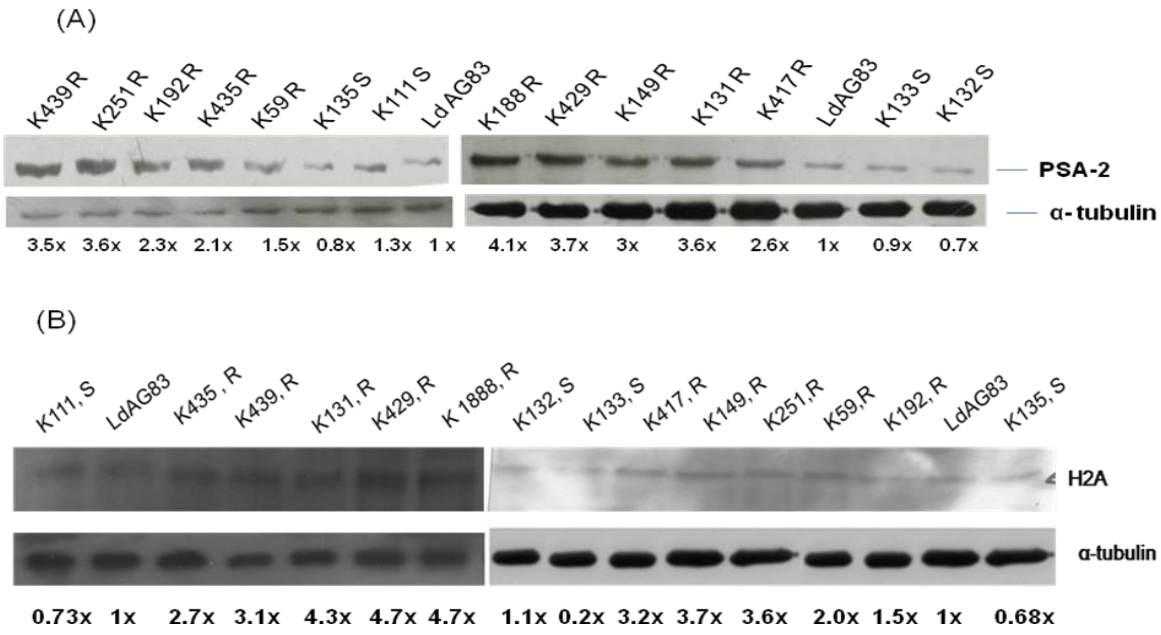


Figure 5.3: Expression analysis of PSA-2 (A) and H2A (B) proteins in *L. donovani* field isolates. 100µg Cell lysates of promastigotes from sensitive (S) and resistant (R) isolates were subjected SDS PAGE using 12% gels, transferred to NC membrane and probed with respective antibodies. The same blot was treated with antibody against α-tubulin protein for normalization. The blots were developed using ECL. The expression of proteins with respect to *Ld*AG83 was expressed in terms of x-fold as analysed by AlphaImager software. PSA-2 and H2A expression fold change with respect to *Ld*AG83 is shown in the term of X -fold.

Discussion:

The mechanism of antimony resistance in the field is largely unknown and most of the information comes from the work on laboratory mutants. The available information on antimony resistance indicates that several mechanisms may coexist in the same cell and different mechanisms may operate in field isolates compared to the lab generated resistant parasites (Singh et al., 2003; Croft et al., 2006). Studies with laboratory generated resistant *Leishmania* cells have revealed several genes which are considered to contribute to antimonial resistance in the parasites, including MRPA, a member of the ABC transporter family, GSH, the enzyme involved in glutathione biosynthesis, and SbIII transporter protein AQP1. Here we analyzed the expression pattern of all three genes in a large number of field isolates for the first time and observed that the expression pattern did not follow the expected pattern in all the field isolates, indicating that alternate mechanisms may operate in the field.

MRPA is reported to be involved in a number of *Leishmania* species selected for resistance to SbIII, SbV or related metal AsIII (Ouellette and Borst, 1991; Haimeur et al., 2000). Its role in resistance was demonstrated by gene transfection (Callahan and Beverley, 1991; Papadopoulou et al., 1994). In a customised DNA microarray studies with 44 known genes involved in drug resistance, the expression of MRPA along with GSH-1 was high in lab generated arsenite and antimony resistant isolate (Guimond et al., 2003). Amplification of the ABC transporter gene MRPA has been reported in an Sb(V) mutant of a *L. guyanensis* cell line (Anacleto et al., 2003) or in axenic amastigotes of *L. infantum* selected for resistance to Sb(III) (El Fadil et al., 2005). However, to date it has remained an open-ended question as to whether similar mechanisms exist in clinical isolates. Study with 4 resistant and 1 sensitive Indian field

isolates showed that MRPA gene was amplified in 3/4 resistant field isolates as part of an extrachromosomal circle. Amplification of MRPA was correlated to increase RNA as determined by real-time PCR (Mukherjee et al., 2007). Higher expression of MRPA was observed in 2 Nepalese resistant field isolates while its expression was low in 2 sensitive isolates (Decuypere et al., 2005). In the present study, 9/10 resistant isolates showed high level of MRPA expression however 1/10 resistant showed low expression level and 1/4 sensitive isolates showed a high expression. Further there was only a low positive correlation with ED_{50} (r=0.45), suggesting that the resistance mechanisms in the field parasites may differ from those of laboratory resistant mutants.

A modulation in thiol levels is correlated with resistance (Decuypere et al., 2005; El Fadili et al., 2005) and is often, but not always, due to an alteration in the expression of GSH1 coding for the rate-limiting step in glutathione biosynthesis. In a recent study heterozygous mutants with one GSH1 allele-inactivated transcribed less GSH1 mRNA and synthesized less glutathione and trypanothione. These mutants were more susceptible to oxidative stresses *in vitro* as promastigotes and showed decreased survival inside activated macrophages in the presence of antimony (SbV), proving the importance of thiols in dealing with oxidants including the action of antimonials (Mukherjee et al., 2009). Overexpression of GSH1 in Arsenite resistant *L. tarentolae* is linked with the SbIII resistance (Grondin et al., 1997). In the present study GSH1 was over-expressed in 8 out of 10 resistant isolates, one resistant isolate expressed GSH-1 comparable to *Ld*AG83 while another resistant isolates showed expression much less (>5 fold lower) than the *Ld*AG83. Increased expression of GSH in majority of the resistant isolate is consistent with the central role that thiols have been proposed to play in antimony resistance in

Leishmania due to their role in the Sb(III) detoxification pathway. However the data suggest the presence of alternate mechanisms in addition, since there was only a moderate correlation of GSH expression with the ED_{50} (r=0.60).

AQP1 gene mediates SbIII uptake and accumulation in the *Leishmania* cells and modulates drug sensitivity when expressed at increased levels. Its over-expression in 3 species of *Leishmania* converted resistant phenotype to sensitive in all (Gourbal et al., 2004). In a study conducted with 2 sensitive and 4 resistant Indian field isolates, AQP1 expression was low in 3/4 resistant parasites while both of the sensitive isolates showed higher expression (Maharjan et al., 2008). In another study with 2 sensitive and 2 resistant Nepalese isolates expression of AQP1 was high in both sensitive and low in both resistant isolates (Decuypere et al., 2005). In the present study, in all sensitive field isolates expression of AQP1 was more than 2.5 fold high compared to *Ld*AG83, however only 4 out of 10 resistant isolates showed the expected low expression while majority (6/10) exhibited no significant change in the expression, resulting in a moderate negative correlation of AQP1 expression with the ED_{50} (r=-0.60). Our data of the expression of AQP1 was similar with the previous studies with clinical isolates where it was concluded that besides AQP1 other factors are responsible for the resistance phenomenon. The resistant phenotype in spite of high AQP1 expression which we observed may be due to increased expression of GSH1 and MRPA in addition to other genes studied here.

A recent proteomic study conducted by Vergenes et al., showed that HSP83 increased drug resistance and reduced drug mediated program cell death activation by interfering with the mitochondrial membrane potential. In the present study, the expression of HSP83 gene was high in 6 out of the 10 resistant isolates, but comparable to sensitive strains in the remaining 4

resistant isolates, indicating that this may be only one of the factors responsible for the resistant phenotype in a fraction of the field isolates.

In our previous study microarray based analysis of gene expression in drug resistant *L. donovani* led to the identification of several transcripts over-expressed in SAG resistant parasites (Salotra et al., 2005). Among all the transcripts that were over-expressed, PSA-2 showed the highest expression (3 fold) indicating that it may play a significant role in mediating drug resistance. PSA-2 represents a family of glycoproteins with leucine rich repeats expressed in both promastigotes and amastigotes forms of *Leishmania* and assists the parasite in invading macrophage by binding through CR-3 receptor (Kedzierski et al., 2004), and resisting complement mediated cell lysis (Brittingham et al., 1995). These studies indicate that PSA-2 plays an important role in the parasite survival and virulence. Recently, another novel leucine-rich repeat (LRR) protein, LinJ34.0570, was implicated in antimony resistance of *Leishmania* since parasites over-expressing this LRR protein were resistant to SbIII as axenic amastigotes and to SbV as intracellular parasites (Genest et al., 2008st). In the present study, the expression of PSA-2 was modulated at both transcript level and protein level, being consistently high in the resistant isolates and low in all sensitive isolates, with a strong positive correlation with ED_{50} , suggesting its role in resistance.

Mitogen-activated protein (MAP) kinase1 which gave a 2 fold higher expression in SbIII resistant isolate in our microarray study (Chapter-4), is a well-known mediator of signal transduction in higher eukaryotes regulating important processes like proliferation, differentiation, stress response and apoptosis. Higher expression of MAPK was observed in all 10/10 of the resistant isolates in the present study while lower expression or comparable

expression with *Ld*AG83 was observed in 3/4 sensitive isolates suggesting the role of MAPK1 in antimony resistance. However markedly high (3.9 fold) higher expression of MAPK 1 in a sensitive isolate suggests that multiple mechanisms of resistance are operating in the field.

Histone synthesis in *Leishmania* is tightly coupled to DNA replication by a post-transcriptional mechanism operating at the level of translation (Abanades, 2009). Very few studies have been carried out on Trypanosomatid histone modifications and on their role in gene regulation. The transcription of histones is regulated by a mechanism coupled to cellular growth. A ten to twelve fold decrease in Histone H4 mRNA levels was observed in parasite cultures from logarithmic phase to stationary phase (Soto et al., 1997). A similar decrease in RNA levels, associated with the growth phase, has been observed for other *L. Infantum* histones, like H2A and H3 (Soto et al.,1992; 1996), however the regulation of *L. major* histone H1 seems to be clearly different to the *Leishmania* core histones (Noll et al., 1997). We have reported the role of histones in antimony resistance in *Leishmania* parasite and observed the preferential expression of histones H1, H2A and H4 in drug resistant *L. donovani* using genomic microarray (Chapter-4). These results impelled us to check the expression of these genes in the natural field isolates to show their association in antimony resistance. In the present study, the 3 histone genes investigated, H1, H2A, and H4 were over expressed in a majority of resistant isolates indicating that histones may play a role in drug resistance among the multiple mechanisms that seem to operate in the field.

Increased expression of both MRPA and HSP83 have been shown to contribute to decreased susceptibility of *Leishmania* parasite not only to SAG but also to Miltefosine and Amphotericin-B, pointing to the phenomenon of cross resistance (Vergenes et al., (2007). Our

studies also revealed cross resistance phenomenon since over-expression of either PSA-2 or H2A (Chapter-6) in SAG sensitive isolates imparted resistance to SAG, accompanied with lower susceptibilty to Miltefosine as well as Amphotericin-B. In the present study both these genes showed consistently high expression in the resistant isolates and low in the sensitive isolates at both RNA and protein level and a strong correlation of expression with drug susceptibility reiterating the fact that these gene products may have a role in a primary mechanism of resistance operative in the field isolates. We recommend expression analysis of PSA-2 and H2A to be included for testing at large scale for establishing a validation assay for distinguishing the resistant and sensitive parasite for monitoring the spread of drug resistance.

Introduction

Resistance to antimonials has emerged as a major hurdle to the treatment and control of VL or KA, the disease caused by *L.donovani* in India, where over 65% of KA-patients are unresponsive to SbV-treatment. Determinants of resistance in laboratory strains are partly known, however the mechanism operating in field isolates is not well understood. Studies are warranted that will provide the understanding of the mechanism of drug resistance and lead to identification of potential drug targets against KA. Presently, two transporters of the ABCC family are described as involved in antimonial resistance. The first one corresponds to PGPA and has been renamed MRPA. The gene ABCC1, coding for MRPA, was found in a number of *Leishmania* species selected for resistance to SbIII, SbV or related metal AsIII.(Haimeur et al., 2000; Ouellette and Borst, 1991). Its role in resistance was demonstrated by gene transfection (Callahan and Beverley, 1991; Papadopoulou et al., 1994). The second ABCC protein was initially isolated by functional cloning while selecting for pentamidine resistance (Coelho et al., 2003). The PRP1 gene was shown to confer cross resistance to antimonials but its localization and the mechanism by which it confers antimonial resistance remains unknown. The role of heat shock proteins (HSP70) in tolerance to antimony has been pointed out using functional cloning that is a powerful strategy to isolate drug resistance genes; although amounts of HSP70 proteins were shown to increase in both cells in contact with antimony and SbIII-resistant mutants (Brochu et al., 2004), transfection of the HSP70 gene does not confer real resistance but rather increase tolerance of the cell to metals. In *L.donovani* strains isolated from India from unresponsive patients, a locus amplified from a region equivalent to chromosome 9 of

Leishmania major was found (Singh et al., 2003). It remains to be found how this locus confers resistance, whether it corresponds to the primary resistance mechanism and whether it occurs frequently in field isolates.

Based on our microarray studies described in chapter-4 and expression studies with field isolates described in chapter-4 we selected two genes H2A and PSA-2 for functional characterization to understand their role in drug resistance/susceptibility. In this chapter, we have attempted to functionally characterize these proteins i.e. H2A and PSA-2 by over-expressing either of the genes in *L. donovani* and studying the effect on growth and phenotype of the parasite.

Materials and Methods

Reagents and Chemicals

SAG (Albert David Ltd,India), MLF (Cayman Chemical Company, USA), AmB, Potassium antimony tartarate [SbIII], RPMI1640, M199, anti rabbit IgG-HRP, anti mouse IgG-HRP , α-tubulin antibody raised in mouse and anti-HA antibody raised in rabbit (Sigma, USA), Fetal Bovine Serum (FBS; Gibco, USA), 8-well Chamber slides (Nunc, USA), Diff-Quik stain solution (DadeBehring, USA), Trizol Reagent, The vector pCR2.1TOPO,Superscript II^{TM} RNase H^{-}Reverse Transcriptase, dNTPs, RNasin, Taq polymerase, Oligo $(dT)_{12–18}$(Invitrogen,USA), DNAse I (Fermentas, USA), RNeasy columns (QIAGEN), SYBR Green PCR Master mix (Applied Biosystem, USA), nitrocellulose membranes, Western blot enhanced chemiluminescent (ECL) detection reagent (Millipore,USA), BioMax MR X-ray film (Kodak), Restriction enzymes (New England Biolabs, USA and MBI Fermentas, USA) were employed for the study.

Cells strains

E. coli strains Top10F' were purchased from Invitrogen, USA. *L. donovani* Sudan (*Ld*1S2D) was a kind gift from Dr. Hira L. Nakhasi, CBER, FDA, USA.

Experimental methods

Over expression of full length Histone H2A or PSA-2 in *L. donovani*

The *Leishmania* expression plasmid pKSNeo (Zhang et al., 1996) was used to express full-length genes of *L.donovani*. The nucleotide sequence of the full length gene was confirmed by cloning

into pCR®2.1-TOPO. Full length insert was used as template using respective gene primers with *SpeI* site and HA tag for sub cloning into pKSNeo, followed by transfection in *L.donovani* cells.

Following steps were carried out for over expression of genes in *Leishmania*:-

I. DNA construction

The DNA encoding full length gene was obtained by PCR from *L.donovani* DNA isolated from the cloned line designated by the World Health Organization as MHOM/SD/62/1S-C12D (LDS 1). It was then sub cloned into pCR®2.1-TOPO TA cloning vector. Ligation was set up as follows.

pCR®2.1-TOPO TA cloning vector (50ng)	1.0 µl
DNA (60 ng)	3.0 µl
Ligation buffer (2X)	5.0 µl
T4 DNA ligase (1U)	1.0 µl
Total	10.0 µl

The ligation reaction mixture was incubated at 14ºC for 16 hrs. *E. coli* TOP 10F' competent cells were prepared and the ligated product was transformed and subjected blue/white + Amp^r selection. The recombinant white Amp^r colonies were screened for the presence of gene of interest by digestion with *EcoRI* enzyme. Further the confirmed colonies were sequenced in an automated sequencer (ABI 3730) using M13 forward and reverse sequence. This recombinant DNA construct was termed pCR®2.1-Histone2A or pCR®2.1-PSA-2.

Competent cell preparation

The host cell culture was streaked on a LB plate from the frozen glycerol stock. A single colony was inoculated into 5ml LB tube. 500µl of the overnight grown culture was further inoculated into 50ml (1:100) LB and allowed to grow for 2-3 hrs until A_{600} reached 0.4-0.5. The culture was chilled on ice, transferred to ice cold 50ml polypropylene tubes and centrifuged at 4000 rpm for 10 mins. The supernatant was decanted and the pellet was resuspended gently in 20ml of ice cold sterile 0.1M $CaCl_2$ and incubated on ice for 20mins. The cells were then centrifuged at 4,000 rpm for 10mins. The pellet was resuspended in 1ml of ice cold 0.1M $CaCl_2$. Chilled glycerol was added to the cells to a final concentration of 20%. About 200µl aliquots were taken for checking the viability, contamination and efficiency of transformation. The rest of the suspension was kept at 4°C for 12-24 hrs to enhance the competency of the cells and then stored in aliquots of 200µl at -70°C.

Transformation of competent cells

150µl aliquot of competent cells was thawed on ice and 1µl of the microarray clone was added to it, mixed by tapping and incubated on ice for 30 mins. The cells were subjected to heat shock at 42°C for 60 secs in a water bath and were immediately chilled on ice for 1-2 min. 850µl of LB was added to the cells and the cells were incubated at 37°C for 1hr in a shaker incubator with constant shaking. The cells were pelleted and resuspended in 100µl of LB and plated on LB agar plates containing 100µg/ml of ampicillin, 10µl of 0.8 M IPTG and 50µl of 20mg/ml of X-gal and incubated at 37°C for 16hrs for selection of recombinant clones (white) over non-recombinant clones (blue).

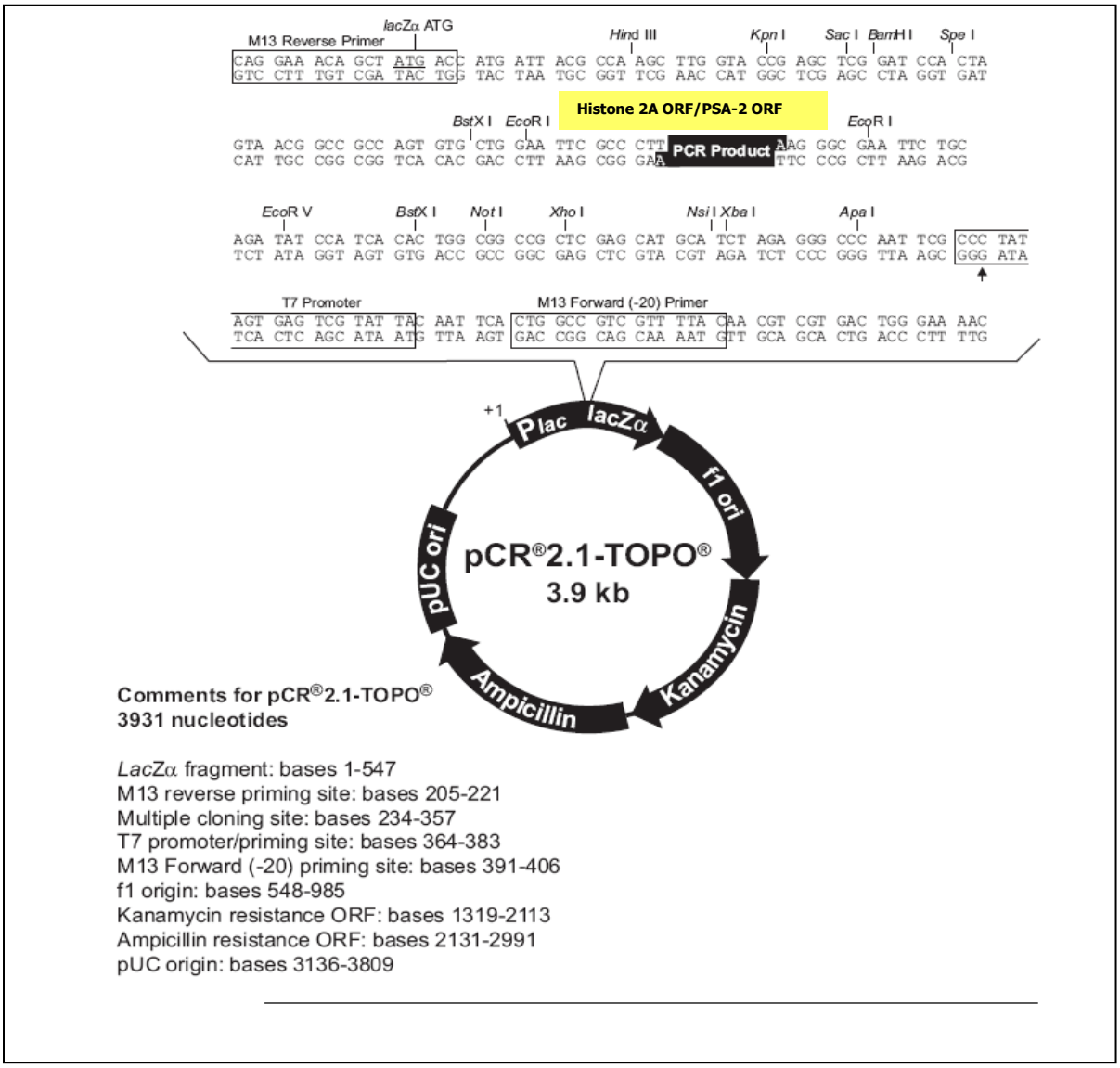


Fig 6.1: Sequence and map of pCR® 2.1 TOPO cloning vector.

II. Insertion of full length gene in pKSNeo vector

Sequence confirmed plasmid DNA of pCR®2.1-TOPO TA -fused with full length insert was used as template using respective gene primers with *SpeI* site and HA tag for sub cloning into the *Leishmania* expression plasmid pKSNeo (Zhang et al., 1996). The vector pKSNeo consists of a 1.6-kb 5' upstream flanking region of the A2 gene, the entire Neo gene and a 1.8-kb fragment containing the A2 3' UTR and a synthetic pyrimidine tract [Zhang *et al.*, 1996]. The sequence and multiple cloning site of the pKSNeo vector is shown in Fig 6.2.

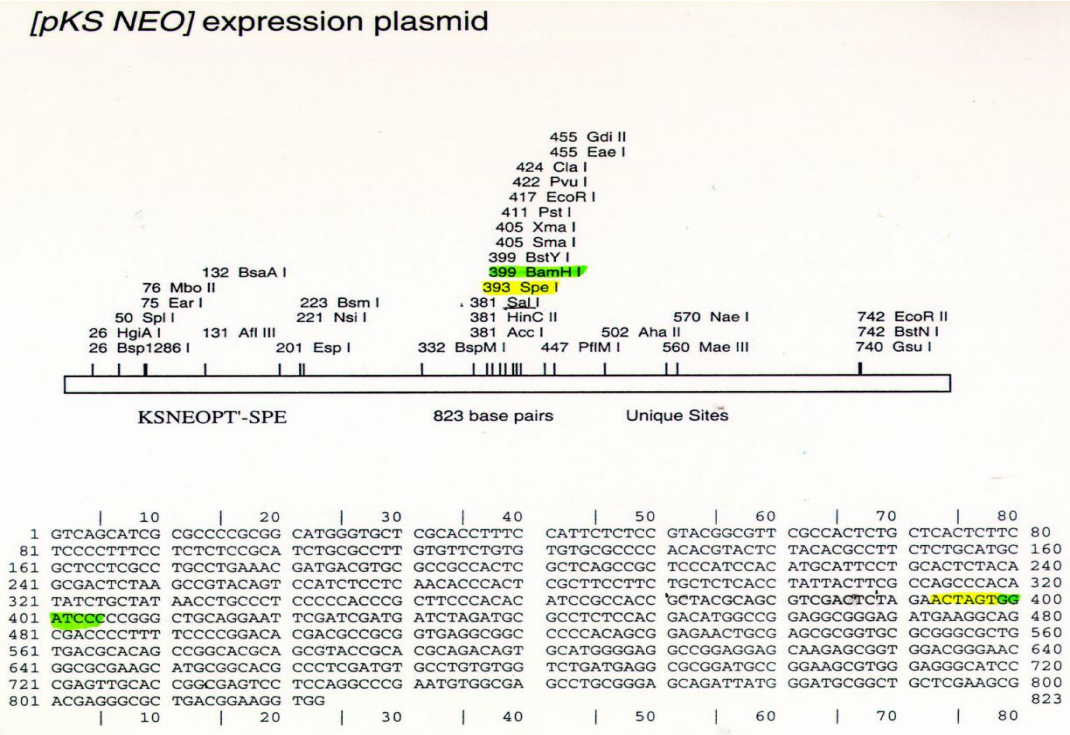


Figure 6.2: The sequence and multiple cloning site of the pKSNeo vector.

These oligos introduced a hemagglutinin tag at the C-terminus of the fusion protein and contained SpeI restriction sites on either end. The SpeI insert was sub cloned into the SpeI site of

pKSNeo, resulting in plasmid constructs that were used to over express wild-type or mutant constructs in *Leishmania* transfectants.

The primer sequence used for the amplification of full ORF of H2A for cloning was: - **Forward Primer**: 5'-GG ACTAGT ATGGCTACTCCTCGCAGCGC-3' and

Reverse Primer: 5' CC A CTA GT C TA C **GCG TAG TCC GGC ACG TCG TAC GGG TA** AGCGCCCGGTGTCG CCTTGC - 3' with *SpeI* site underlined and HA tag in bold.

Similarly the primer sequence used to amplify PSA-2 was as follows:

Forward: 5' GG A CTA GT A TGG CGC TGT GCG TGC GTC GGC -3'

Reverse: 5' CC A CTA GT C TA C **GCG TAG TCC GGC ACG TCG TAC GGG TA**C GCC GCC AGCCCC ACG CTC AG- 3' with *SpeI* site underlined and HA tag in bold.

The amplified products were ligated using T4 DNA ligase using pKSNeo vector and transformed in *E. coli* TOP 10F' cells. The recombinant Amp^r colonies were screened for the presence of gene of interest by digestion with *SpeI* enzyme. The orientation of the gene of insert was checked by digestion with *HindIII* and *XhoI*.

III. Restriction digestion

Plasmids were prepared from bacterial colonies containing the respective constructs along with pKSNeo vector and digested with *SpeI* enzyme to release the insert.

Plasmid (pKSNeo + Insert)	5.0 µl
SpeI enzyme (1U)	1.0 µl
NEB buffer 2	1.0 µl

H_2O	3.0 µl
Total	10.0 µl

E. coli cells containing plasmids with correct inserts were grown in bulk and plasmids were isolated using Endofree plasmid isolation kit (Qiagen) following manufacturer's instructions.

IV. Transfection of construct in *L.donovani*

a) Preparation of cold DNA

20µg plasmid DNA was re-precipitated in 3M sodium acetate and 2 volumes of isopropanol, washed with 80% ethanol, dried and dissolved in 20µl sterile TE buffer and the plasmid was kept in ice until transfection.

b) Electroporation

The construct (20µg) was transfected into one lab strain (LdS) and one field isolate (K133). $2X10^8$ mid log phase *L.donovani* cells were harvested, washed once with electroporation buffer and resuspended in ice-cold electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mMNa_2HPO_4,6mM glucose, pH7.4). 0.5 ml cells were taken in 2mm cuvette and 20µl cold plasmid DNA was added to it for transfection. The DNA was electroporated into *Leishmania* cells at 450 V, 500µF, 800π resistance. Following electroporation, cells were recovered in M199+10%FCS for 24hrs. After 24hrs, cells were washed once with M199 and resuspended in M199 + 10% FCS + 20µg/ml G418 (selectable marker). The drug concentration was increased to a final concentration of 50µg/ml for the selection of mutant cell lines.

V. Western blot analysis

Western blot analysis was performed as described in chapter 4.

VI. *In vitro* assay for drug sensitivity

The parasite isolates were analyzed for *in vitro* drug susceptibility as described in chapter 3. Briefly, Mouse-macrophage–adherent cell line J774A.1 ($2X10^5$ cells/well) in 8-well chamber slides was infected with stationary-stage promastigotes at a 10:1 (*L.donovani*: macrophage) ratio and was incubated in 5% CO_2 for 4 h at 37ºC. After washing, the cells were incubated for 12–18 h. Infected cells were reincubated, for 48 h, with SAG (0,3, 10, 30, 60 and 100 µg/mL), MLF (0,0.5,1.25,2.5,5,10,30µg/ml) or AmB (0, 0.25,0.5,1.0,2.0,3.0,4.0 µg/ml). After staining with Diff-Quik solutions, the numbers of amastigotes per cell were counted in 100 macrophages. The percent killing was calculated by sigmoidal regression analysis (Origin6.0; Origin Lab).

Results:

Cloning of Histone2A/PSA-2

PCR was carried out with specific primers for the amplification of Histone2A ORF/PSA-2 ORF from *L. donovani* genome. The reaction yielded a ~480bp product with *L. donovani* DNA for H2A and 1158 bp product for PSA-2 (Figure 6.3 and 6.5). The PCR product was cloned into pCR®2.1-TA cloning vector. The presences of gene Histone2A/PSA-2 were confirmed by digestion with *EcoRI* enzyme which yielded a product of 480bp/~1.1kb respectively (Fig. 6.4 and 6.6).

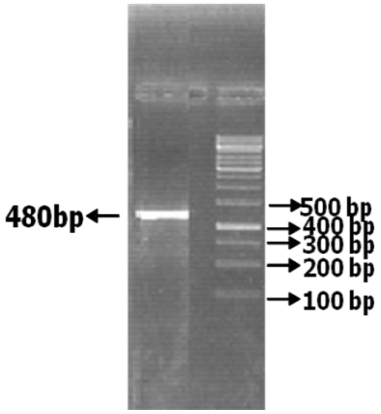


Fig.6.3. PCR amplified Histone2A ORF

Lane 1: PCR product

Lane 2: Molecular weight marker

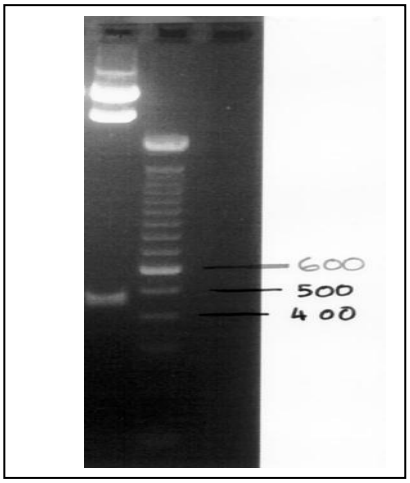


Fig.6.4. *EcoRI* digestion showing the presence of 480bp Histone2A ORF.Numbers on right indicate sizes in bp.

Lane 1: *EcoRI* digestion of pCR®2.1 + H2A plasmid
Lane 2: Molecular weight marker.

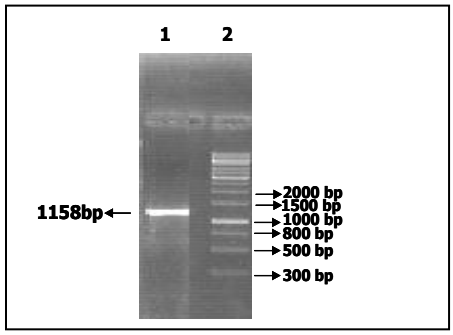


Fig.6.5. PCR amplified PSA-2 ORF

Lane 1: PCR product

Lane 2: Molecular weight marker

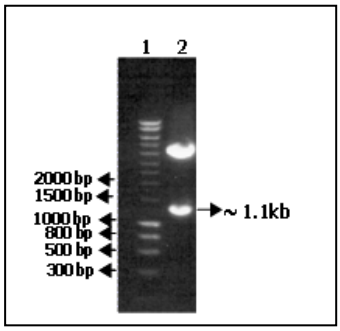


Fig.6.6. *EcoRI* digestion showing the presence of 1158bp PSA-2 ORF

Lane 1: Molecular weight marker

Lane 2: *EcoRI* digestion of pCR®2.1 + PSA-2 plasmid

Cloning of Histone 2A /PSA-2 gene in *Leishmania* expression vector

The Histone H2A/PSA-2 gene was cloned in pKS Neo vector for overexpressing H2A/PSA-2 in *L. donovani*. The recombinant plasmids were selected and the presence of insert was confirmed with digestion with *SpeI* enzyme. Further correct orientation was confirmed by digestion with *HindIII* and *XhoI*.

Transfection in *L. donovani*

To see whether over-expression of H2A/PSA-2 would impart a drug resistance phenotype in a drug sensitive parasite, we over-expressed H2A/PSA-2 with a HA tag in two SAG susceptible *L.donovani* isolates, *LdS* and K133.

H2A/PSA-2 gene was transfected in *L donovani* and the mutant cell lines containing pKS-H2A^{++} / pKS-PSA-2^{++} were selected under G418 pressure. pKS Neo expression vector was used as control in the transfection experiments. The transfected parasites were selected and the over-expression was validated by western blotting of the total

promastigote lysate probed with either anti HA antibody (Fig 6.7) showing the expression of the exogenous protein or anti-*Ld*H2A Ab/ anti-*Ld*PSA-2 Ab (Fig 6.8 A and B) showing both the endogenous and the exogenous proteins simultaneously. The expression of H2A was 5 to 6 fold higher in *LdS*H2A^{++} and K133H2A^{++} in comparison to controls, while the expression of PSA was 6 to 7 fold higher in *LdS*PSA-2^{++} and K133PSA-2^{++} in comparison to controls.

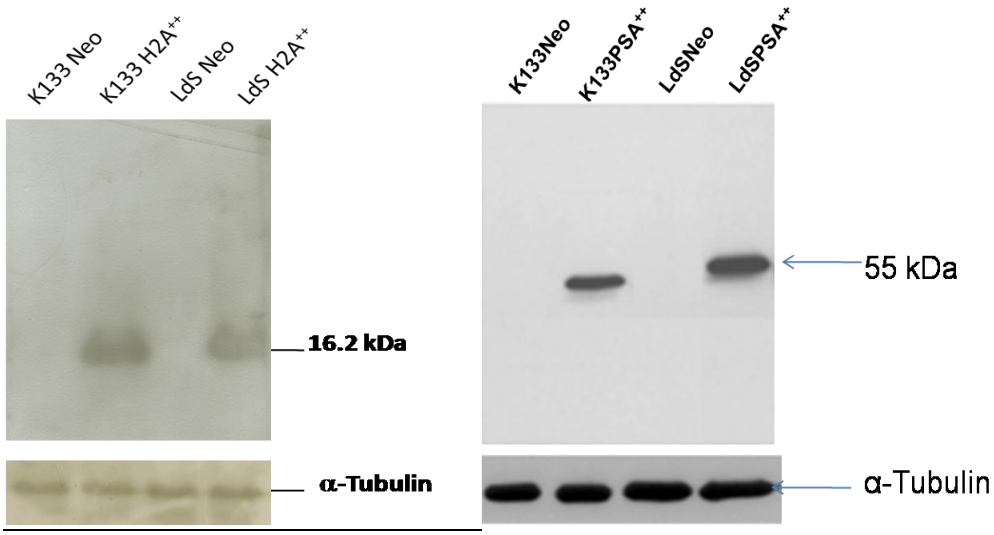


Fig 6.7 Western blot using anti HA antibody to confirm the presence H2A (16.2 kDa) and PSA-2 (55 kDa) overexpression in *Leishmania donovani.* 100µg total promastigotes lysates were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membranes. Membrane was probed with anti HA antibody followed by rabbit IgG conjugated with HRP and developed by using ECL. The blot was rebound with an α-tubulin antibody to monitor the amount of protein lysates loaded on the gel.

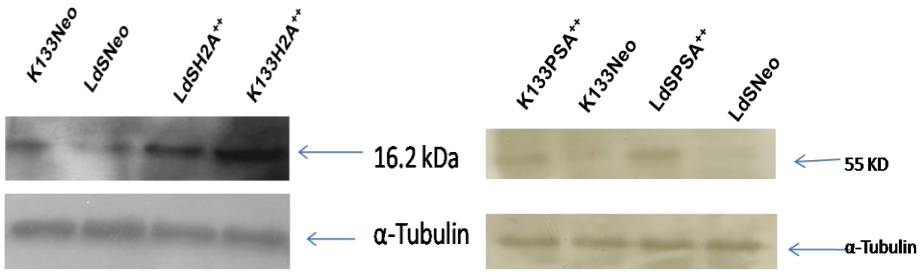
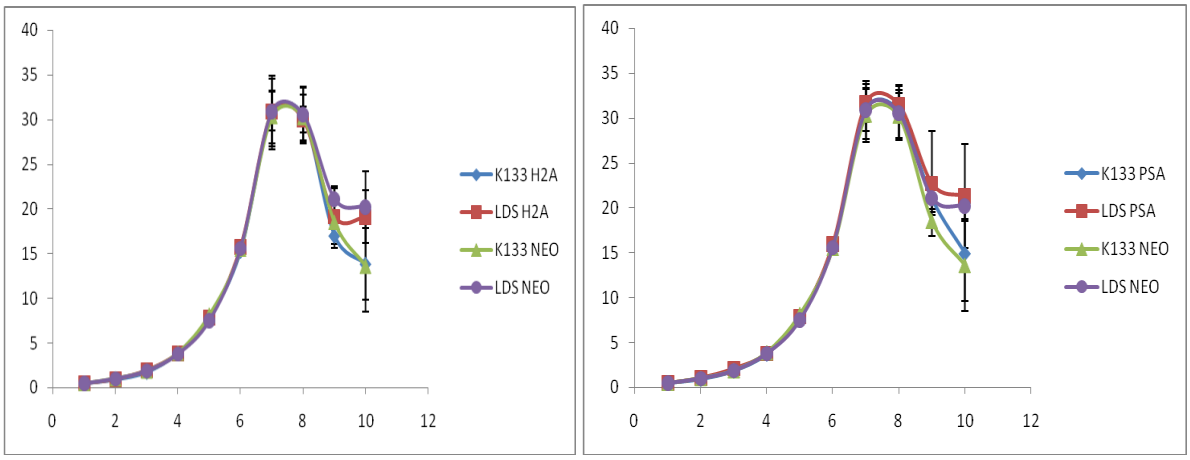


Fig 6.8 Western blot analysis for endogenous and exogenous protein expression (A) using anti H2A antibodies and (B) using antiPSA-2 antibodies in parasites over-expressing H2A /PSA-2 compared with control. Other details same as in figure 5.7.

Effect on growth

The growth of the transfected parasite *Ld*SH2A^{++} and K133H2A^{++} as well as *Ld*SPSA-2^{++} and K133PSA-2^{++} , monitored as promastigotes over a period of 10 days, was comparable to the growth of controls transfected with the plasmid alone indicating that over-expression of H2A or PSA-2 had no effect on promastigote viability [Fig 6.9 (A) and (B)].



(A) **(B)**

Fig. 6.9 Growth curve of *LdH2A*++ (A) and *Ld*PSA-2++(B). X-axis of the graph represents number in days while Y-axis represents number of parasite in millions. Log phase promastigotes were diluted to 0.5 $X10^5$ cells/ml in 5 ml cultures to allow cells to enter logarithmic phase. For each time point, the parasites were pelleted by centrifugation, washed with PBS, and counted by light microscopy. The growth pattern was studied in triplicate and the value shown is the mean ± SD of the three values.

***In vitro* drug susceptibility of the mutant *L.donovani* cell lines**

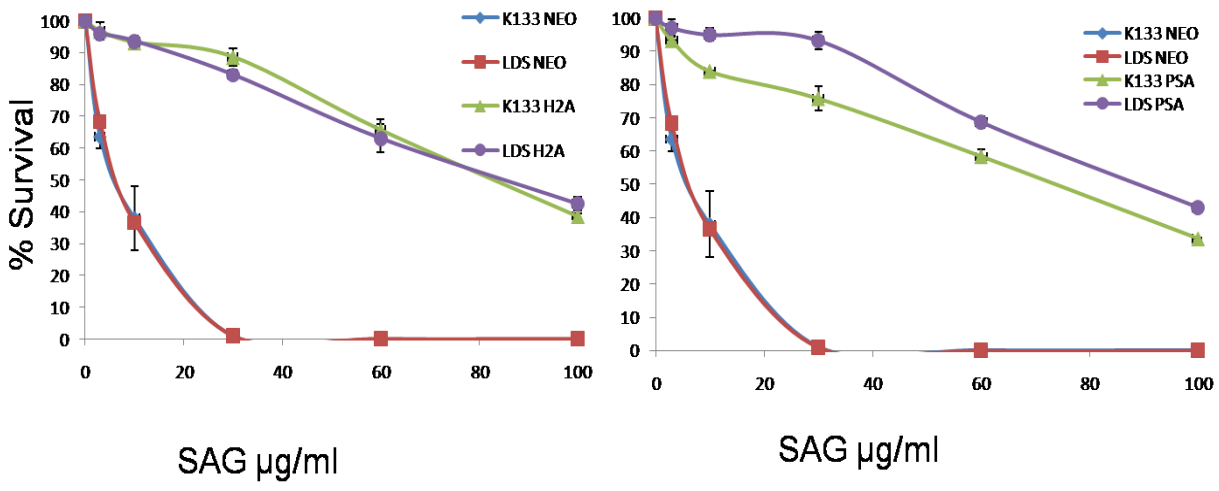
Initially assessment of infectivity by counting parasites infected macrophages per 100 macrophages indicated similar infectivity of *Ld*S$H2A^{++}$, $K133H2A^{++}$, *Ld*S$PSA\text{-}2^{++}$ and $K133PSA\text{-}2^{++}$ with respect to controls.

Next the parasites over-expressing H2A/PSA-2 were analysed for their susceptibility towards 3 different anti-leishmanial drugs (SAG, MLF and AmB) as amastigotes assays.

The ED_{50} of $K133H2A^{++}$ *and* *Ld*S$H2A^{++}$ for SAG was 79.31± 2.47 µg/ml and 82.15 ± 7.12 µg/ml respectively, which was significantly higher ($p<0.001$) than their corresponding controls

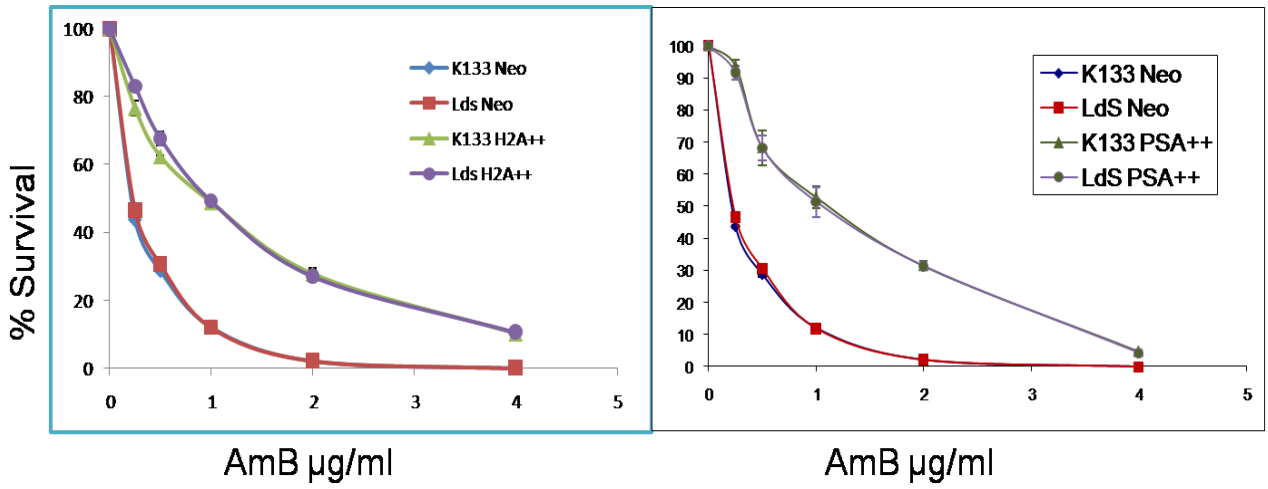
K133 Neo (ED_{50} 6.28 ± 0.92 µg/ml) and *LdS* Neo (ED_{50}6.37 ± 0.26 µg/ml) (Fig. 6.10 A). Overexpression of H2A decreased susceptibility of parasites not only towards SAG but also for AmB and MLF. The ED_{50} of K133H2A^{++} *and LdS*H2A^{++} for AmB was 0.89 ± 0.007 µg/ml & 0.96 ± 0.01 µg/ml which was significantly higher ($p<0.001$) than the controls K133 Neo (0.24 ± 0.007 µg/ml) and *LdS* Neo (0.265± 0.007 µg/ml) (Fig. 6.11B, table 6.1). ED_{50} for MLF was significantly higher ($p<0.05$) for K133H2A^{++} (2.91 ± 0.26 µg/ml) and *LdS*H2A^{++} (2.66 ± 0.45 µg/ml) than respective controls (1.4 ± 0.25 µg/ml and 1.46 ± 0.19 µg/ml), (Fig. 6.12 C, Table 6.1).

The ED_{50} of K133PSA-2^{++} *and LdS*PSA-2^{++} for SAG was 70.19 ± 3.56 µg/ml and 80.57 ± 5.01 µg/ml respectively, which was significantly higher ($p<0.001$) than their corresponding controls K133 Neo (ED_{50} 6.28 ± 0.92 µg/ml) and *LdS* Neo (ED_{50}6.37 ± 0.26 µg/ml) (Fig. 6.10A). Similar to the results with H2A, overexpression of PSA-2 decreased susceptibility of parasites not only towards SAG but also for AmB and MLF. The ED_{50} of K133PSA-2^{++} *and LdS*PSA-2^{++} for AmB was 1.11 ± 0.11 µg/ml & 1.08 ± 0.07 µg/ml which was significantly higher ($p<0.001$) than the controls K133 Neo (0.24 ± 0.007 µg/ml) and *LdS* Neo (0.265± 0.007 µg/ml) (Fig. 6.11B, Table 6.1). ED_{50} for MLF was significantly higher ($p<0.05$) for K133PSA-2^{++} (3.53 ± 0.14 µg/ml) and *LdS*PSA-2^{++} (3.95 ± 0.11 µg/ml) than respective controls (1.4 ± 0.25 µg/ml and 1.46 ± 0.19 µg/ml), (Fig. 6.12C, Table-6.1).



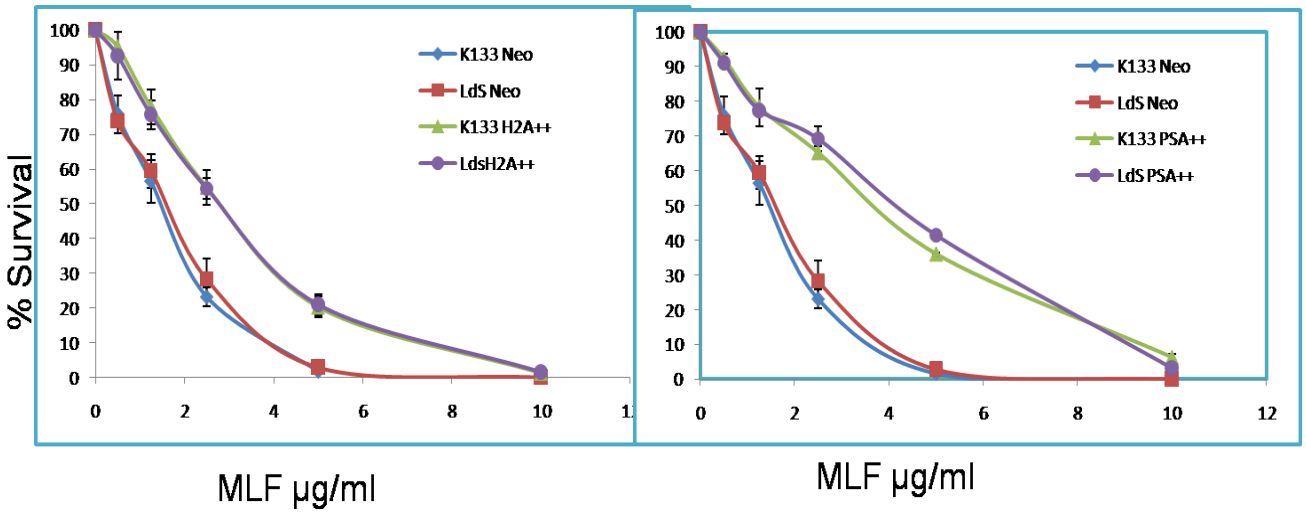
(A) (B)

Figure 6.10 Drug susceptibilities of mutant parasites towards SAG (A) *Ld*S H2A^{++} and K133 H2A^{++} (B) *Ld*S PSA^{++} and K133 PSA^{++}.



(A) **(B)**

Figure 6.11 Drug susceptibilities of mutant parasites towards AmB (A) *Ld*S H2A^{++} and K133 H2A^{++}. (B) *Ld*S PSA^{++} and K133 PSA^{++}.



(A) **(B)**

Figure 6.12. Drug susceptibilities of mutant parasites towards MLF (A) *Ld*S H2A^{++} and K133 H2A^{++}. (B) *Ld*S PSA^{++} and K133 PSA^{++}.

Table 6.1: ED_{50} (µg/ml) of transfected parasites *LdSH2A*$^{++}$, *K133H2A*$^{++}$, *K133PSA-2*$^{++}$ *LdSPSA-2*$^{++}$ *to* SAG, AmB and MLF.

Parasite ID	ED_{50} SAG	ED_{50} AmB	ED_{50} MLF
K133 Neo	6.28 ± 0.92	0.24 ± 0.007	1.40± 0.25
*Ld*S Neo	6.37 ± 0.26	0.265 ± 0.007	1.46 ± 0.19
K133 H2A^{++}	79.31 ± 2.47	0.89 ± 0.007	2.91 ± 0.26
*Ld*S H2A^{++}	82.15 ± 7.12	0.96 ± 0.01	2.66 ± 0.45
K133 PSA-2^{++}	70.19 ± 3.56	1.11 ± 0.11	3.53 ± 0.14
*Ld*S PSA-2^{++}	80.57 ± 5.01	1.08 ± 0.07	3.95 ± 0.11

ED_{50} to each of the 3 drugs for each strain of the parasites based on sigmoidal regression analysis by Origin 6.0 software. Each data point represents the mean ± SD of 3 separate assays.

Discussion:

SAG has been the first drug of choice against Leishmaniasis and resistance to this drug is a major problem in the field primarily in the Indian subcontinent, but also throughout the world (Lira et al., 1999; Singh et al., 2006). Apart from first line drug antimonials other available drugs in India are Amphotericin B (AmB) as well as its liposomal formulation, Ambisome, and the oral drug Miltefosine (MLF). Both AmB and MLF are highly effective in antimony resistant patients but suffer from limitations of adverse reactions and cost. In a phase III trial of MLF, Sundar et al (2002) observed 94% cure rate for MLF in comparison to 97% cure rate in AmB treated group. A recent report of unresponsiveness to Ambisome in Sudanese patients of VL is worrisome and indicate emergence of AmB resistant parasites (Mueller et al.,2007). Preliminary data from a phase IV trial in India involving domiciliary treatment with miltefosine and weekly supervision suggested doubling of the relapse rate, indicating somewhat lower drug efficacy than in Phase II and III trials and providing warning that drug resistance could develop quickly warranting plans to prevent it (Sundar and Murray, 2005; Croft et al.,2006).

The increase in resistance to SAG has led to an upsurge in therapeutic failure, and with the limited chemotherapeutic alternatives, understanding the mechanisms responsible for the resistance could help lead to effective drug treatment strategies. In the present study we selected two genes namely H2A and PSA-2; both of them highly expressed at RNA and protein level in resistant *L.donovani* parasites, and over-expressed

in two sensitive *L.donovani* to investigate if they alter the phenotype from sensitive to resistant. *Leishmania* parasites possess core histones H2A, H2B, H3, H4 (Soto et al., 1992, 1997) and linker histone H1 (Fasel et al., 1993; Martínez et al., 2002; Papageorgiou et al., 2002) that facilitate the formation of higher order chromatin structures. In eukaryotes, posttranslational modifications of core histones act in diverse biological processes such as gene regulation, DNA repair and chromosome condensation (mitosis). Histone synthesis in *Leishmania* is tightly coupled to DNA replication by a post-transcriptional mechanism operating at the level of translation (Abanades et al., 2009). Very few studies have been carried out on Trypanosomatid histone modifications and on their role in gene regulation. Recently, a CHIP-Chip assay revealed that H3 histones at the origins of polycistronic transcription of protein-coding genes are acetylated and possibly modification in the acetylation state of these origins regulates transcription initiation (Thomas et al., 2009). However, the role of histone proteins in antimony resistance has not been established, in our study of gene expression in drug resistant *Leishmania donovani* by DNA microarrays, of the 11 DNA clones up-regulated in resistant parasites four were corresponding to histones namely H1, H2A and H4. In *Leishmania,* histone mRNA levels are regulated by a mechanism coupled to cellular growth. During promastigote growth, histone H1 mRNA progressively accumulates from early log phase to stationary phase. Addition of histone H1 affects chromatin condensation of parasite nuclei and its over-expression in *Leishmania* has resulted in the reduced infectivity of the parasite *in vitro* as well as *in vivo* (Smirlis et al.,2006; Masina et al., 2007). We observed 1.5 fold upregulation of histone H1 at the RNA level in a lab

generated resistant isolate and more than 6 fold upregulation at the protein level (Chapter-4).

We observed up to 2 fold changes in expression of core histones H2A and H4 by microarray and similar results were obtained by RT-PCR. The expression of H2A was found to be elevated at the both RNA and protein level in a number of field isolates of *Leishmania* signifying its functional role. Unlike histone H1, decrease in RNA levels, associated with the growth phase, has been observed for other *L. infantum* histones, H2A, H3 and H4 (Soto et al.,1992, 1996, 1997). We used the identical growth points for comparative expression profiling eliminating the possibility of growth related differential expression of histones. Transfection studies using overexpression of a gene have proved their utility in several studies dealing with assessment of *Leishmania* phenotypes. Overexpression of HSP83 in the sensitive *L.donovani* isolate conferred increased resistance to trivalent antimony as well as AmB and MLF (Vergnes et al., 2007). Overexpression of P299 gene in the sensitive *L.donovani* isolate conferred increased resistance to trivalent antimony and miltefosine (Choudhury et al., 2008). Here, we showed that transfection of histone H2A in 2 sensitive isolates of *L. donovani* conferred more than 12 fold increase in SAG resistance. In addition, it also conferred resistance to other anti-leishmanial drugs AmB and MLF albeit slightly lower than SAG (~4 fold and 2 fold respectively) in comparison to the wild type.

The surface molecules of the promastigote are involved in the steps following inoculation leading to successful invasion of macrophages. A substantial amount of

information has shown the lipophosphoglycan family of glycolipids, major parasite receptor for macrophages (Handman and Goding, 1985) and major surface molecule, gp63**,** a parasite protease similar to the zinc-dependent thermolysin family of enzymes (Bouvier et al., 1989; Chaudhuri et al., 1989; Ip et al**.,** 1990) to be the major molecules of the promastigote involved in the interactions with the mammalian host. Apart from lipophosphoglycan and gp63, the only other characterized surface molecules include several transporter-like molecules such as an ATPase (Meade et al**.,** 2003), and the family of surface glycoproteins termed the promastigote surface antigen-2 (PSA-2) complex (Kahl and McMahon-Pratt, 1987; Murray et al., 1989).

The promastigote surface antigen-2 (PSA-2) complex were shown to be related to the *L. amazonensis* protein gp46/ M2 and comprises a group of immunogenic surface antigens linked to the surface of the *Leishmania* promastigote with glycosylphosphatidylinositol anchors (Lohman et al., 1990). The *L. major* genome contains at least 14 PSA- 2 genes on a 950-kilobase chromosome and comprising -20% of the length of this chromosome (Ivens et al., 2005). PSA-2 genes appear to be transcribed in a complex manner with multiple RNAs. The complex genomic organization of PSA-2 genes is present in other members of the genus suggesting that PSA-2 function is important for the biology of *Leishmania.* We tested the expression of PSA-2 protein in a number of antimony resistant and sensitive *Leishmania* using anti PSA-2 antibodies. We found higher expression of PSA-2 protein in all the antimony resistant parasites with respect to sensitive parasites, further higher expression was also

observed for PSA-2 at RNA level (Chapter-4).Towards understanding the role of this surface protein in drug resistance, PSA-2 was overexpressed in *Leishmania donovani* and its effect in parasite phenotype was determined. Here, we showed that transfection of PSA-2 in 2 sensitive isolates of *L. donovani* conferred more than 12 fold increase in SAG resistance. In addition, it also conferred resistance to other anti-leishmanial drugs such as AmB and MLF albeit slightly lower than SAG (~4 fold and 2 fold respectively) in comparison to the wild type. Finally, our results suggest that overexpression of H2A or PSA-2, if observed in the field isolates, will indicate not only the resistance to antimonials but also lower susceptibility for Amphotericin B and Miltefosine, especially in India where plausible cross resistance seems to operate in field isolates (Kumar et al., 2009).

The demonstration that a higher expression of the nucleosome core histone H2A or PSA-2 is found in clinically isolated SAG resistant parasites and forced overexpression of this protein in susceptible strains converts them to resistant suggests a mechanism of resistance not previously recognized. The well established role of histone H2A in DNA packaging opens up a new area of investigation to overcome drug resistance and develop more effective anti-leishmanials. PSA-2 antigen is considered a virulence gene since it elicits Th1type immune response (Handman et al., 1995). In the present study, we have shown that the PSA-2 gene has a role in resistance not only to SAG but also to AmB and MLF. In conclusion we successfully established the role of H2A and PSA-2 in the development of resistance to major drugs currently in use against VL.

Conclusions and Future Scope of Work

In the present study, microarray experiments were carried out to compare gene expression in drug sensitive and resistant *Leishmania donovani*. Two genes that showed significantly higher expression at both RNA and protein level in a number of resistant field isolates of *L.donovani* were selected for further characterization.

1. Histone 2A (H2A).
2. Parasite Surface Antigen-2.

Parasites cultures were set up from bone marrow aspirates of KA patients (n=19), including 4 SAG responsive and 15 non responsive cases. In search of antimony resistance related genes, the gene expression profiling was undertaken using highly sensitive microarray technology. We developed a microarray chip for *L. donovani* comprising of 8448 PCR amplified inserts (1.0 to 1.5 kb) from genomic library clones, 24 positive and 12 negative controls, each printed in duplicate. Two clinical isolates of *L.donovani,* K80 and K135 were used for the microarray study on the basis of their antimony susceptibility. The isolate K80 was adapted as SbIII resistant parasite (K80SbIII) by *in vitro* passages with a stepwise increase in the concentration of SbIII from 10 to 125 µg/ml (K80SbIII). Microarray experiment was performed between antimony sensitive (K135 with ED_{50} for SAG 4.22 ± 0.38 µg/ml) and resistant (K80SbIII; ED_{50} above 100 µg/ml). We identified several genes that were uniquely expressed or showed altered expression in the drug resistant strain, indicating their potential role in drug resistance. Genes coding for Histone 1 (H1), Histone 2A (H2A), Histone 4 (H4), MAP-kinase

(MAPK), and two hypothetical proteins were transcribed more abundantly in the antimony resistant parasite in comparison to the sensitive parasite, while genes encoding amino acid transporter showed consistent over-expression in the sensitive parasite. Differential expression of all of these genes was validated by semi-quantitative reverse transcriptase (RT)-PCR assay and at protein level by western blotting for H1, H2A and MAPK.

We examined the expression of various genes putatively involved in antimony resistance (AQP1, GSH, MRPA, HSP83, PSA2 reported in earlier studies and MAPK, Histone H1, H2A and H4 identified in our study) in natural Sb(V)-resistant (n=10, range ED_{50} =11.82±1.39µg/ml to 20.30 ± 0.84µg/ml) and sensitive (n=4, range ED_{50} =3.45 ± 0.28 µg/ml to 5.63 ± 0.57 µg/ml) *L.donovani* parasites, K80SbIII and one standard isolate of *L. donovani Ld*AG83 using Real Time PCR. Expression of Sb(III) transport protein AQP1 was up-regulated in all the sensitive isolates by 2.5 fold to 7.5 fold indicating the increased uptake of antimony and thereby the higher drug susceptibility of sensitive isolates compared to resistant isolates. In resistant isolates AQP1 expression was comparable to *Ld*AG83 in 6 of 10 while in other 4 it was 1.79 to 6.25 fold less. Expression of GSH was down regulated in all the sensitive isolates and it was 1.86 to 13.45 fold high in majority of resistant isolates. Expression of MRPA was high by more than 1.9 fold in 8/10 resistant parasites and ranged up to 10 fold in comparison to *Ld*AG83. Expression of MRPA was comparable with *Ld*AG83 in 3/4 sensitive isolates.

Expression analysis of the new target molecules identified in transcriptomic analysis revealed interesting results in the field isolates. Expression of all three histones H1, H2A and H4, PSA-2 and HSP83 was down regulated or comparable to *Ld*AG83 in all 4 sensitive isolates while MAPK showed low expression in 3/4 sensitive isolates. We observed >1.5 fold high expression

of MAPK in 10/10, PSA-2 and H1 in 9/10, H2A in 8/10, H4 in 7/10 and HSP83 in 6/10 resistant isolates with respect to *Ld*AG83.

Further the expression was analysed at protein level with available antibodies, we observed higher expression of H2A and PSA-2 protein in all SAG resistant isolates, though H1 showed comparable protein expression in resistant and sensitive isolates. Based upon these results we have attempted to see the effect of over-expression of H2A and PSA-2 on the growth and phenotype of one WHO standard sensitive *L.donovani* isolate (*L. donovani* Sudan, *Ld*S) and one sensitive field isolate (K133). The growth of the parasites was not affected by the over-expression of either of the gene H2A/PSA-2. Forced over-expression of either of this protein in susceptible strains converted the phenotype to resistant suggesting a mechanism of resistance not previously recognized. In conclusion we successfully established the role of H2A and PSA-2 in the development of antimony resistance in *L.donovani*.

Future scope of work

In our study, we have identified a few resistance related genes of which characterization of two genes has been carried out. Other differentially expressed genes identified such as MAP kinase and Histone 1 need to be investigated and characterized to determine their role in antimony resistance. Further, the mechanism of development of drug resistance by PSA-2 and H2A genes needs to be explored.

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Appendix

Preparation of reagents

10X MOPS

0.2M MOPS pH 7.0, 20mM Sodium acetate, 10mM EDTA pH 8.0

Filter sterilized with 0.45μm filter.

DEPC water

0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37°C it was autoclaved.

100X Denhardt's solution

Ficoll400	10g
Polyvinylpyrrolidone	10g
Bovine serum albumin Fraction V	10g
dH_2O	500ml

Filter sterilized and stored at 4°C.

20X SSC

175.3gm of NaCl, 88.2gm of Sodium citrate were dissolved in 800ml distilled water. pH was set to 7.0 with HCl. Volume was adjusted to 1ltr, aliquots prepared and the solution was sterilized by autoclaving.

Preparation of bacterial culture media

LB medium (Luria Broth)

10 gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride (Hi Media) were dissolved in 1liter of dw. Media were sterilized by autoclaving for 20 minutes at 15 lb/sq.in.

LB Agar

15gm of agar powder, 10gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride, (Hi media) were dissolved in dw. Media were sterilized by autoclaving for 20 minutes at 15lb/sq.in. LB agar was allowed to cool to 45°C and poured (~30 ml per plate) in 90 mm disposable petri plates (Tarsons) along with appropriate antibiotics and allowed to solidify.

Antibiotics

Ampicillin

100mg/ml ampicillin stock was prepared in autoclaved dw and sterilized by filtration through 0.22 μm filter. 100μl aliquots were stored by freezing at –20°C.

Kanamycin

50mg/ml kanamycin stock was prepared in autoclaved dw and sterilized by filtration through 0.22 μm filter. 100μl aliquots were stored by freezing at –20°C.

Chloramphenicol

34 mg/ml chloramphenicol stock was prepared in ethanol and stored at –20°C. Chloramphenicol is required to ensure the presence of pLysS.

Stock solution of commonly used reagents

1M Tris

121.1gm of Tris base was dissolved in 800ml of dw and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-2H_2O was added in 800ml of dw, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

3M sodium acetate

204.5gm of $C_2H_3O_2Na$. 3H_2O was dissolved in 400ml of dw, pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS

10gm of electrophoresis grade SDS was dissolved in 70ml of dw, heated at 60°C to dissolve and the volume made up to 100ml.

Ethidium Bromide (10 mg/ml)

10mg of ethidium bromide was dissolved in 1ml dw, stored in a opaque bottle.

30% Acrylamide Stock

29.2gm of acrylamide and 0.8gm of bis-acrylamide were dissolved in 50ml of ddH_2O. Volume was made up to 100ml, the solution filtered through Whatman no. 1 paper, degassed and stored in an opaque bottle.

Calcium Chloride (0.1 M)

1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of ddH_2O and sterilized by autoclaving.

IPTG (1M)

238mg of IPTG was dissolved in 1ml of dw, filter sterilized and stored at -20°C in 50µl aliquots.

Coupling Buffer (0.2 M)

0.42 gm of $NaHCO_3$ was dissolved in 40 ml, pH was set to 9.0 and volume made upto 50 ml. Sterilized using 0. 45µm filter

Sodium Phosphate (1M)

Monobasic

138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of dw and volume made up to 1liter.

Dibasic

268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of dw and volume made up to 1liter.

Ammonium persulfate (10%)

To 1gm of ammonium persulfate, 10ml of dw was added and the solution stored for several weeks at 4°C.

10 X TAE buffer (Tris acetate, EDTA)

4.84gm of Tris base in 80ml of dw was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

10X TBE buffers (Tris borate, EDTA)

8gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA.\ H_2O$ were dissolved in 700ml dw and the final volume made up to 1 liter.

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of dw. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

SDS-PAGE electrophoresis buffer

3gm of Tris base, 14.4gm of glycine and 1gm of SDS were dissolved in 1 liter of dw.

Protein transfer buffer

5.8gm of Tris base, 2.9gm of glycine and 0.33gm of SDS were dissolved in 500ml of dw. 200 ml of ethanol was added and the final volume was made up to 1 liter.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

Tris-Cl (pH6.8)	100mM
DTT	200mM
SDS	4%
Bromophenol blue	0.2%
Glycerol	20%
β-mercaptoethanol	10%

10X Ligation buffer

Tris.Cl pH 7.8	500mM
$MgCl_2$	100mM
DTT	100mM
ATP	10mM

10 X Amplification buffer

Tris.Cl pH 8.3	100mM
$MgCl_2$	15mM
KCl	500mM
Gelatin	0.1%

DNA loading dye (6X)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved dw.

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

30% acrlyamide solution	4.0 ml
1.5M Tris-Cl pH 8.8	2.5 ml
dw	3.3ml
10% SDS	100µl
10% APS	100µl

TEMED	10µl

Composition of stacking gel (5%) (5.0 ml)

30% acrylamide solution	0.83ml
1.0M Tris.Cl pH 6.8	0.68 ml
dw	3.4ml
10% SDS	50µl
10% APS	50µl
TEMED	5µl

Staining solution

1gm of coomassie blue was dissolved in 450ml of methanol. 100ml of glacial acetic acid was added and the volume made up to 1liter by double distilled water, filtered through Whatman no.1 filter and stored at room temperature.

Destaining solution

Methanol: water: acetic Acid were mixed in the ratio of 45:45:10 and stored at room temperature.

PSGEMKA Buffer

20µl of 1M Na-Phosphate, 20.8 ml of 1M NaCl, 400µl of 250mM Glucose, 10µl of 0.5M EDTA, 20 ml of 10mM $MgCl_2$, 20 ml of 100mM KCl, 0.02% BSA buffer added to 138ml of autoclaved distilled water.

Electroporation Buffer

0. 25 gms HEPES, 0.403 gms NaCl, 186mg KCl, 6.2mg Na_2HPO_4, 54 mg glucose, pH7.4 added to 100 ml dw and sterilized by filtration.

Publications and Presentations

Publications:

1. **Dhiraj Kumar**, Ruchi Singh, Arpita Kulshrestha, Narendra Singh Negi, Poonam Salotra. Increased expression of Histones and PSA-2 in antimony resistant Indian *Leishmania donovani*: Potential biomarkers of drug resistance. **(Communicated)**
2. Ruchi Singh, **Dhiraj Kumar**, Robert Duncan, Hira Lal Nakhasi, Poonam Salotra. Over expression of Histone H2A modulates drug susceptibility in *L. donovani* parasites. **International Journal of Antimicrobial Agents. 2010; 35: 50-57.**
3. V Ramesh, Joginder Kumar, **Dhiraj Kumar**, Poonam Salotra. A retrospective study of intravenous sodium stibogluconate alone and in combinations with allopurinol, rifampicin and an immunomodulator in the treatment of Indian post-kala-azar dermal leishmaniasis. **Indian Journal of Dermatology, Venereology, and Leprology. 2010; 76:36-42.**
4. **Dhiraj Kumar,** Arpita Kulshrestha, Ruchi Singh, Poonam Salotra. *In vitro* susceptibility of field isolates of *Leishmania donovani* to Miltefosine and Amphotericin B: correlation with SAG susceptibility and implications in the endemic area. **Antimicrobial Agents and Chemotherapy. 2009; 53: 835-838.**
5. **Dhiraj Kumar,** V Ramesh, Sandeep Verma, M Ramam, Poonam Salotra. Post-kala-azar dermal leishmaniasis (PKDL) developing after treatment of visceral leishmaniasis with amphotericin-B and miltefosine. **Annals of Tropical Medicine and Parasitology, 2009; 103: 727-730.**
6. **Dhiraj Kumar,** Gurumurthy Srividya , Sandeep Verma , Ruchi Singh , Narendra Singh Negi, Konstantina Fragaki, Joanna Kubar and Poonam Salotra. Presence of anti Lepp12

antibody: a marker for diagnostic and prognostic evaluation of Visceral Leishmaniasis. **Transactions of the Royal Society of Tropical Medicine and Hygiene 2008; 102:167-171.**

7. Singh R, **Dhiraj Kumar**, Ramesh V, Negi N S, Singh S, Salotra P. Visceral Leishmaniasis, or Kala Azar(KA): High incidence of refractoriness to antimony is contributed by anthroponotic transmission via Post-KA Dermal Leishmaniasis. **Journal of Infectious Diseases 2006; 194:302-306**.
8. Tanu Rana, Shashank Gupta, **Dhiraj Kumar**, Sharad Sharma, Manish Rana,Vikram S. Rathore, Ben M.J. Pereira. Toxic effects of pulp and paper-mill effluents on male reproductive organs and some systemic parameters in rats. **Environmental Toxicology and Pharmacology 2004; 18:1–7.**

Manuscript under Preparation:

9. Arpita Kulshrestha, Ruchi Singh, **Dhiraj Kumar**, Poonam Salotra. Susceptibility of Indian isolates of *Leishmania donovani* towards Paromomycin and Sitamaquine**.**

Abstracts in Proceedings:

1. **Dhiraj Kumar**, Ruchi Singh, Arpita Kulshrestha, N.S. Negi, Poonam Salotra **"**Differential expression of Histones, HSP83, MAPK and PSA-2 contributes to drug resistance in field isolates of *Leishmania donovani* in **"Keystone Symposia on "Drug Discovery for Protozoan Parasites" held at Breckenridge, Colorado(USA), March 22-26, 2009. P58**
2. **Dhiraj Kumar** , Ruchi Singh , Robert Duncan, Hira L Nakhasi , Poonam Salotra" Transcriptome analysis of antimony resistant and sensitive isolates of *L. donovani* isolated from Indian patients of Kala-azar**" In National Conference on Emerging Trends in Life Sciences Research held at BITS PILANI, India, March 6-7, 2009. P1.**

3. Ruchi Singh, **Dhiraj Kumar**, Robert Duncan, Hira L Nakhasi, Poonam Salotra "Transcriptome profiling for identification of antimony resistance determinants in *Leishmania donovani* isolated from Indian patients of Kala azar**". In World *Leishmania* Congress 4 at Lucknow, India, Feb 3rd to 7th 2009. P169**

4. Arpita Kulshrestha, Ruchi Singh, **Dhiraj Kumar**, Poonam Salotra " Susceptibility of Leishmania donovani clinical isolates from India towards Paromomycin and Sitamaquine. **In World *Leishmania* Congress 4 at Lucknow, India, Feb 3rd to 7th 2009. P162.**

5. **Dhiraj Kumar,** Arpita Kulshrestha, Ruchi Singh, Poonam Salotra. *In vitro* susceptibility of Indian Kala-azar isolates to Miltefosine and expression analysis of markers for drug resistance. **In 9th Sir Dorabji Tata Symposium on "Antimicrobial Resistance", held at Bangalore, India, March 10-11, 2008.P 73.**

6. Singh R, **Dhiraj Kumar,** Duncan R, Nakhasi HL, Salotra P. Transcriptome profiling for identification of antimony resistance determinants in *Leishmania donovani* isolated from Indian patients of Kala azar. **In IX International Symposium on "Vectors and Vector Borne Diseases" held at Puri, Orissa, India Feb 15 – 17, 2008.P 54.**

7. P. Salotra, K. Padamkumar, **Dhiraj Kumar**, A. Kulshrestha**,** R. Singh. Challenges in the treatment of Visceral Leishmaniasis: Potential of marine natural products as alternative drug candidates. **In Keystone symposium on "Drugs against protozoan parasites", held at. California, USA, Jan28- Feb1, 2007, P 52.**

8. P. Salotra, K. Padamkumar, **Dhiraj Kumar**, A. Kulshrestha, R. Singh. Potential of marine natural products as alternative drug candidates. In National Symposium on **"Current trends in the development of herbal drugs" and "27th Annual conference of Indian Association of Biomedical Scientists (IABMS)" held at Trivandrum, Kerala, Nov25-27, 2006. P 79**.

Chapter in Book/Periodicals:

1. **Dhiraj Kumar**., Kulshrestha, A., Singh, R., **Salotra, P**. In vitro susceptibility of Indian kala-azar isolates to Miltefosine and expression analysis of markers of drug resistance. In "**Antimicrobial Resistance- the Modern Epidemic: Current Status and Research Issues**", **Eds D. Raghunath, Nagaraja V and C.D. Rao, Macmillan publishers India Ltd. (2009). PP 406-407.**
2. V. Ramesh, **Dhiraj Kumar**, Poonam Salotra. Antimonial Therapy in Post-kala-azar dermal Leishmaniasis-A Hobson's choice. In **"Drugs and Pharmaceuticals- Current R & D Highlights" Eds S. Tandon and S. Mehrotra, Publisher Central Drug Research Institute, Lucknow. (Oct-Dec 2008) PP 7-10.**

Presentations:

1. **Dhiraj Kumar** , Ruchi Singh, Arpita Kulshrestha , N.S. Negi , Poonam Salotra **"**Differential expression of Histones, HSP83, MAPK and PSA-2 contributes to drug resistance in field isolates of *Leishmania donovani* in **"Keystone Symposia on "Drug Discovery for Protozoan Parasites" held at Breckenridge, Colorado (USA) March 22-26, 2009.P58**
2. **Dhiraj Kumar** , Ruchi Singh , Robert Duncan, Hira L Nakhasi , Poonam Salotra" Transcriptome analysis of antimony resistant and sensitive isolates of *L.donovani* isolated from Indian patients of Kala-azar**" In National Conference on Emerging Trends in Life Sciences Research held at BITS PILANI, India March 6-7,2009. P1.**
3. Ruchi Singh , **Dhiraj Kumar** , Robert Duncan , Hira L Nakhasi , Poonam Salotra. "Transcriptome profiling for identification of antimony resistance determinants

in *Leishmania donovani* isolated from Indian patients of Kala azar**". In World *Leishmania* Congress 4 at Lucknow, India, Feb 3rd to 7th 2009. P169.**

4. **Dhiraj Kumar,** Arpita Kulshrestha, Ruchi Singh, Poonam Salotra. *In vitro* susceptibility of Indian Kala-azar isolates to Miltefosine and expression analysis of markers for drug resistance. **In 9th Sir Dorabji Tata Symposium on "Antimicrobial Resistance", held at Bangalore, India, March 10-11, 2008. P 73.**

Brief Biography of the Candidate

Name	Dhiraj Kumar
Date of Birth	14 Feb' 1979
Educational qualification	

Examination Passed	**Board/University**	**Percentage**	**Year of passing**
B.Sc Biotechnology	Patna University, Patna.	66.5	2001
M.Sc Biotechnology	IIT-Roorkee	CGPA 7.17 on scale of 10	2003

Academic Achievements

1. **Awarded Bill and Melinda Gates Foundation Keystone Symposia Global Health Award** to attend the Keystone symposia "Drug Discovery for Protozoan Parasites" held at Beaver Run Resort, Breckenridge, Colorado, USA on March 22- March 26, 2009.
2. Awarded **Best Poster Presentation** at IX-Sir Dorabji Tata Symposium on Antimicrobial Resistance organized by Sir Dorabji Tata centre for Research in Tropical Diseases and in association with Department of Microbiology and Cell Biology, Indian institute of Science held on 10^{th} to 11^{th} March 2008.
3. Awarded **Senior Research Fellowship** by Council of Scientific and Industrial Research (CSIR), India (August 2006).
4. Qualified **Graduate Aptitude Test in Engineering (GATE)** for award of fellowship to pursue research in engineering field with 85.48 percentile. (2004).
5. Qualified Joint **CSIR-UGC** National examination leading to award of Junior research fellowship to pursue research (December 2003).

6. Qualified All India entrance exam conducted by Indian Institute of Technology Roorkee for admission to post graduation in Biotechnology (2001) with **All India Rank 18.**
7. **Scholarship for M.Sc.** from **Department of Biotechnology**, Govt.of India.(2001-2003)

Conferences Attended

1. Attended Keystone Symposia on "Drug Discovery for Protozoan Parasites" held at Breckenridge, Colorado (USA), March 22-26, 2009.
2. Attended National Conference on "Emerging Trends in Life Sciences Research" held at BITS PILANI, India, March 6-7, 2009.
3. Attended "World *Leishmania* Congress 4" at Lucknow, India, Feb 3rd to 7th 2009.
4. Attended IX-Sir Dorabji Tata Symposium on "Antimicrobial Resistance" organized by Sir Dorabji Tata Centre for Research in Tropical Diseases and in association with Department of Microbiology and Cell Biology, Indian institute if Science held on 10th to 11th March 2008.
5. Interactive Meet with European and Indian Science Icons organized by Department of Science & Technology, Embassy of Germany in New Delhi, EU Delegation of EC in India, held at New Delhi on 8th February, 2007.
6. Attended 21st annual conference of Indian Association of Pathologists and Microbiologists, Delhi Chapter organized by Institute of Pathology (ICMR) and Safdarjung Hospital and V.M.M. College held on 16 April 2006.
7. International Workshop on Education and Capacity Building in Biophysics: Needs of the Asian African Region. Organized by the Department of Biotechnology, Indian Institute of Technology Roorkee held on 24-25 February 2003.

8. National symposium on Biophysics Organized by the Department of Biotechnology, Indian Institute of Technology Roorkee, held on 21-23 February 2003.

Work Experience

June – July 2002

Underwent a two months training under the supervision of Dr. R. Madhubala at School of Life Sciences, Jawaharlal Nehru University, New Delhi on developing Serodiagnosis methods for Leishmania donovani. During the tenure I had learnt techniques for expression, and purification of recombinant protein using column chromatography and ELISA.

July 2004 to July2006

Working as **CSIR JRF** at Institute of Pathology (ICMR) under Dr. Poonam Salotra. My PhD involves the "Studies of gene expression in drug resistant *Leishmania donovani* by DNA Microarrays".

July 2006 to April 2009

Working as **CSIR SRF** at Institute of Pathology (ICMR) under Dr. Poonam Salotra. My PhD involves the "Studies of gene expression in drug resistant *Leishmania donovani* by DNA Microarrays".

April 2009 to till date

Working as SRF (Senior Grade) at Institute of Pathology (ICMR) with Dr. Poonam Salotra in a European Commission Project entitled "New tools for monitoring drug resistance and treatment response in Visceral Leishmaniasis in the Indian subcontinent".

Brief Biography of the Supervisor

Personal Particulars

- Name Poonam Salotra
- Designation : Deputy Director
- Place of work : Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi – 110 029, India.
- Tel. No: 26198402, 26166124
- Fax No: 26166124
- E. mail: salotra@vsnl.com, salotrap@icmr.org.in

Academic Qualifications

- B.Sc. Hons (Chemistry) from Delhi University in 1974.
- M.Sc. (Biochemistry) from P.G.I., Chandigarh in 1976.
- Ph.D. from V.P. Chest Institute, Delhi University in 1980.

Professional Appointments:

- Postdoctoral Fellow at Roche Institute of Molecular Biology, Nutley, New Jersey, U.S.A. (1980).
- Research Officer at Tuberculosis Research Centre, Madras (1982).
- Principal Investigator under Young Scientists scheme of Department of Science & Technology at Centre for Biotechnology, J NU, New Delhi (1991).
- Pool Research Officer (CSIR) at Centre for Biotechnology, JNU, New Delhi (1993).
- Senior Research Officer at Institute of Pathology (ICMR), New Delhi (1996).
- Assistant Director at Institute of Pathology (ICMR), New Delhi (2000).
- Deputy Director at Institute of Pathology (ICMR), New Delhi (2005 till date)

Research Interests:

- Worked mainly on the molecular basis of pathogenesis of infectious diseases such as Kala-azar, Anthrax, Tuberculosis and Cholera. Currently working on development of diagnostic tests and attenuated vaccines for kala-azar, mechanism of drug resistance in Indian kala-azar, and characterization of immune responses in patients of kala-azar and post kala-azar dermal leishmaniasis.

Awards/Honours

1. Basanti Devi Amir Chand Award conferred by ICMR in 2007.
2. Prof. BK Aikat Award conferred by Indian Council of Medical Research conferred by ICMR in 2007.
3. ICMR International fellowship for Senior Biomedical Scientists for the year 2006.
4. Awarded Courtesy Fellowship by CBER, FDA, USA in Sep 2005.
5. Awarded fellowship by National Foundation of Infectious Diseases, USA in 2005.
6. Granted ICMR Award for Excellent Research output in July 2004.
7. Silver Jubilee award by Indian Association of Medical Microbiology in 2003.
8. Awarded Courtesy Fellowship by CBER, FDA, USA in Dec 2003
9. Kshanika Oration Award, a National award for Eminent Woman Scientist, conferred by Indian Council of Medical Research in 2002.
10. National Science Talent Scholarship awarded by N.C.E.R.T. , New Delhi

Patents

1. Awarded US Patent No. 6,855,522, in 2005, for "Species-specific PCR assay for detection of *Leishmania donovani* in clinical samples of kala-azar and post kala-azar dermal leishmaniasis".
2. US patent no. 20060240046 for "Live attenuated *Leishmania* vaccines"

Membership of professional associations

1. Society for Parasitology, India (Life member
2. Society of Biological Chemists, India (Life member).
3. Association of Clinical Biochemists of India (Life member)
4. Indian Association of Medical Microbiologists, India (Life member
5. Indian Immunology Society, India (Life member).

Publications

Publications in indexed foreign journals	57
Publications in indexed Indian journals	05
Publications in Proceedings	41
Chapter in Books	03
Total	106