Chapter 1: Introduction and Review of Literature
1.0 Overview

Leishmaniasis, constitutes a major public health problem with increasing pattern of disease burden (Desjeux, 2001; Desjeux, 2004). It is a neglected tropical disease (NTD) which affects mainly the poorest population groups, primarily in rural areas. According to ranking after malaria it is a second most prevalent parasitic disease. Leishmaniasis has been considered as a tropical affliction that constitutes one of the six entities on the list of most important diseases of World Health Organization/Tropical Disease Research (WHO/TDR) viz. Malaria, Schistosomiasis, Filariasis, Chagas disease, African Trypanosomiasis, Leishmaniasis, Leprosy, Tuberculosis. Leishmaniasis is endemic in 88 countries, causing considerable morbidity and mortality (Desjeux, 1996; WHO, 1999). Among them 66 countries are in the old world and rest 22 are in the new world with an estimated incidence of 1-1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis. The population at risk is estimated at 350 million people, with an overall prevalence of 12 million. Of the 500,000 new cases of VL, which occur annually, more than 90% are reported from India, Bangladesh, southern Sudan and northeast Brazil (Desjeux, 1999).

Leishmaniasis, a vector borne parasitic disease caused by obligate intramacrophage protozoan is characterized by its diversity and complexity. A total of about 21 leishmania species have been identified to be pathogenic to human (Herwaldt, 1999). In most instances they cause disease in animals, and humans become infected incidentally when they enter in the area of endemicity. Numerous rodent and canine have been incriminated as reservoirs. Though several animal reservoirs have been identified in different countries for leishmaniasis, no animal reservoir is identified yet in India. It is presumed that skin lesions of a late sequel of the visceral form called post- kala-azar dermal leishmaniasis (PKDL) acts as reservoir in this case.

There are 500 species of phlebotomine species, of these about 30 species of the female Phlebotomus belonging to 6 genera are suspected or proven vectors transmitting parasites from animal to animal, animal to man, and man to man (Shaw, 1994). The new world leishmaniasis vector is Lutzomyia longipalpis. On rare occasions, transmissions also occur congenitally or as a result of blood transfusions (Bora, 1999; Singh and Sivakumar, 2004). The clinical manifestations of leishmaniasis depend on complex
interactions between the virulence characteristics of the infecting *Leishmania* species and the immune responses of its human host. The result is a spectrum of diseases ranging from localized skin lesions to diffuse involvement of the reticuloendothelial system. Human leishmaniasis consists of mainly two clinical forms, simple self-healing cutaneous leishmaniasis (CL) and; disfiguring and debilitating or even fatal visceral leishmaniasis (VL). In addition to the two major clinical forms of the disease- VL and CL, there are other cutaneous manifestations including mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), recidivans leishmaniasis (RL) and post-kala-azar dermal leishmaniasis (PKDL) that are often associated with the host immune status. Though all forms can have devastating consequences; visceral leishmaniasis (VL) also known as kala-azar (Kapoor et al., 2008) is the most severe form of the disease, which if untreated, has a mortality rate of 100%.

Unfortunately, there is still lack of effective, affordable and easy-to-use drugs for leishmaniasis treatment. Since vaccine against leishmaniasis is still under development (Brandonisio and Spinelli, 2002), the control lies solely on chemotherapy. However, emergence of drug resistance in parasitic protozoa is becoming a major public health problem. The monitoring of antimonial resistance is a crucial issue in the anthroponotic focal region of leishmaniasis in India (Sundar et al., 2001; Croft, 2001). The higher-dosage requirement for effective treatment of PKDL, with increasing number of treatment failures in India, indicate that anthroponotic transmission may be involved in the spreading of drug-resistant *L. donovani* as a possible cause of unresponsiveness to SAG. With this objective in view, the present study is focused on the *L. donovani* isolates from patients with either Visceral leishmaniasis (VL) or Post-kala-azar dermal leishmaniasis (PKDL) who were from Bihar region with zones of varying leishmaniasis endemicity. In summary, this thesis deals with molecular characterization and validation of biomarkers of antimony resistance in the *Leishmania donovani* VL and PKDL field isolates.

2.0 History of Leishmaniasis

Kala-azar was first noticed in Jessore in India in 1824, when patients suffering from fever thought to be due to malaria failed to respond to quinine. By 1862, the disease had spread to Burdwan, where it reached epidemic proportions. In 1901, William Leishman
identified certain organisms in smears taken from the spleen of a patient who had died from 'dum-dum fever'. Initially, these organisms were considered to be trypanosomes, but in 1903 Captain Donovan described them as being new. The link between these organisms and KA was eventually discovered by Major Ross, who named them *Leishmania donovani*.

3.0 Present status of disease

3.1 Geographic distribution

Leishmaniasis is a world-wide vector borne disease, affecting 88 countries: 72 are developing countries and 13 of them are among the least developed. More specifically VL occurs in 65 countries. Leishmaniasis has a worldwide distribution with important foci of infection in Central and South America, Southern Europe, North and East Africa, the Middle East and the Indian subcontinent (Figure 1). The majority (90%) of VL cases occur in poor rural and suburban areas of 5 countries: Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 1999). Cutaneous leishmaniasis is present in at least 82 countries. Ninety percent of all CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (Desjeux, 1999). Ninety percent of all mucocutaneous leishmaniasis (MCL) occur in Bolivia, Brazil and Peru. The largest focus of PKDL is in the India and Sudan where

* 350 million people at risk * 88 countries affected
* 12 million people infected* 72 developing countries

Figure 1: Global distribution of leishmaniasis (WHO, 1990)
causative organism is *Leishmania donovani*.

### 3.2 Leishmaniasis: Indian sub-continent

VL typically strikes people in rural villages, often in the poorest regions and among the people least able to afford treatment. In the Indian sub-continent: India, Bangladesh and Nepal account about 400,000 new cases of leishmaniasis every year. It is estimated that about 200 million people are at risk of VL but this is a gross underestimation (Desjeux, 1996; Singh et al., 2006). India is one of the world's hotbeds of VL. In India, leishmaniasis is commonly referred to as *Kala Dukh* (Black Misery) or Kala-azar (Black Fever). It is caused by *Leishmania donovani*. The disease has spread from four districts to roughly 36 districts of Bihar and 10 districts of West Bengal. While the disease is present predominantly in the districts adjoining the Ganges, an ever-increasing trend is evident however; sporadic cases have also been reported in north-west, in the states of Himachal Pradesh, Punjab, foothills of Himalayas, Jammu and Kashmir. Sporadic cases were also reported from Tamil Nadu. In 2006, in India 38,656 cases of VL were officially reported: in Bihar (29,711), Jharkhand (7,211), West Bengal (1,628), Uttar Pradesh (80) and few imported cases in Delhi. It has been observed that KA cases had peaks and troughs but admission of PKDL cases in dermatology department of Patna medical College (PMC) increased linearly (Thakur and Kumar, 1992).

### 4.0 The parasites

### 4.1 Morphology and life cycle

*Leishmania* belongs to the family trypanosomatidae, which has adapted to heterogeneous environment e.g. from ambient temperature in sandfly gut to 37 °C temperature in mammalian host and from neutral pH in sandfly stomach to highly acidic in macrophage phagolysosomes. All *Leishmania* species are morphologically similar and display two main developmental stages through their life cycle: the amastigotes that reside inside the reticuloendothelial cells of the vertebrate host and the promastigotes that replicate in the gut of a phlebotomine sandfly. The life cycle starts when a parasitized female sandfly takes a blood meal from a human host (Figure 2). Briefly, the female sandfly picks up infected cells with its blood meal; amastigotes are then released in the midgut of the insect and are transformed to the procyclic stage where they start multiplying actively without penetrating the hemocoele.
Elongated and flagellated promastigotes migrate to cardiac valve from midgut epithelium where they transform into short, spherical and non-dividing promastigotes. High densities of infectious parasites block the cardiac valve at the digestive tract of the sandfly and as the insect swallows the blood from the host, it expels the valve's content including the parasites. Within the human host, the parasites are phagocytosed by reticuloendothelial cells and this fosters their metamorphosis, reproduction and survival. They profusely replicate inside the reticuloendothelial cells until the cell eventually bursts. The released parasites infect other phagocytic cells spreading within the mammalian host. When another insect bites the infected vertebrate host, it swallows infected macrophages with the parasite and the cycle starts again.

5.0 Clinical features
Clinical spectrum of leishmaniasis is shown in Figure 3.

5.1 Visceral leishmaniasis (VL)

Figure 2: *Leishmania* life cycle in mammalian host and sand fly vector (adapted from http://www.dpd.cdc.gov/dpdx).
Visceral leishmaniasis is distributed in South and Central America, Africa, Mediterranean region, Indian sub-continent and China. VL is the most severe form (nearly always fatal, if left untreated), characterized by undulating fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathies and anemia. Prothrombin production is reduced causing mucosal hemorrhage. It causes large scale epidemics with high fatality rate. VL is caused by \textit{L. donovani} in the Indian subcontinent and in East Africa. Visceral leishmaniasis co-infection with human immunodeficiency virus (HIV) cases is also increasing now a day probably due to human migration and resettlement.

5.2 **Cutaneous leishmaniasis (CL)**

CL is also known as oriental sore. It is frequently self-healing in the Old World but, when the lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma. Its more severe form, Recidivans leishmaniasis, is very difficult to treat, long lasting, destructive and disfiguring (WHO, 1999). Infection by parasites of the \textit{L. major}, \textit{L. tropica} and \textit{L. aethiopica} complexes of species (Old World) and of the \textit{L. mexicana} and \textit{L. braziliensis} complexes (New World) usually gives rise to CL.

5.3 **Mucocutaneous leishmaniasis (MCL)**

Also known as “Espundia”, it causes extensive destruction of oral-nasal and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face and great suffering for life. MCL is mostly related to \textit{Leishmania} species of the New World (America) such as \textit{L. Mexicana}, \textit{L. braziliensis} and \textit{L. guyanensi}.

5.4 **Diffuse cutaneous leishmaniasis (DCL)**

This occurs in individuals with defective cell mediated immune response. Its severity is due to disseminated lesions that resemble those of lepromatous leprosy, which never heal spontaneously and is subject to relapse after treatment with any of the currently available drugs. Because of the devasting consequences to the patient, it is recognized as a special health problem. DCL is caused by \textit{L. aethiopica} and \textit{L. amazonensis} (Desjeux, 1996; WHO, 1999).

5.5 **Post-kala-azar dermal leishmaniasis (PKDL)**

Post-kala-azar dermal leishmaniasis is one of the most neglected entities in the world at the moment. PKDL is a dermatropic form of leishmaniasis developed by part of the treated VL patients (WHO, 1990) previous known history of VL (el-Hassan et al., 1992).
The disease is generally incurable but not fatal and constitutes a residual reservoir. The disease is characterized by the development of macules, papules and nodules, which first appear around the mouth and if do not heal spontaneously, they become denser and spread over the entire body (Berman, 1997). PKDL caused by *L. donovani*, occurs in nearly 10–20% of the patients cured of VL in India and in about 50% of the patients cured of VL in Sudan (Zijlstra et al., 1994; Ramesh and Mukherjee, 1995; Zijlstra et al., 2003). In India, the disease occurs between one and 20 years after recovery from VL while in Sudan, it develops during or within months after treatment of VL (Ramesh and Mukherjee, 1995; Zijlstra et al., 2003). PKDL cases are important reservoir for continuation of cycle of this disease which resurges in epidemic form every 15-20 years in endemic areas of Bihar, Bengal, Assam, and Eastern UP.

6.0 Current Status of Chemotherapy

The current situation for the chemotherapy of leishmaniasis is more promising than it has been for several decades with both new drugs and new formulations of old drugs; either recently approved or in clinical trial (Croft and Yardley, 2002; Croft and Coombs, 2003).
The chemical structures of the commonly used drugs are given in Table 1. In recent years four new potential therapies have been introduced for visceral leishmaniasis (Table 1). These include amphotericin B liposome formulation registered in the United States and Europe (AmBisome) (Berman et al., 1998; Meyerhoff, 1999); oral miltefosine (Sundar et al., 2002) which has been approved for use in visceral leishmaniasis in India; a parenteral formulation of aminosidine (paromomycin) (Thakur et al., 2000) registered in India and in phase IV clinical trials in India (www.iowh.org); in East Africa (www.dndi.org) and oral sitamaquine (previously WR6026), which has completed phase II trials in India, Kenya, and Brazil (Dietze et al., 2001; Jha et al., 2005; Wasunna et al., 2005) and is being developed by GlaxoSmithKline (http://science.gsk.com/about/disease.htm). Treatment of CL has also improved by introduction of topical formulations of paromomycin (el-On et al., 1992; Soto et al., 2002; Asilian et al., 2003).

6.1 Antimonials

Pentavalent antimonial compounds are still the mainstay of the treatment for both VL and CL. The commercial scale production of Pentostam (sodium stibogluconate) and Glucantime (meglumine antimoniate) was carried out by GlaxoSmithKline, UK and Aventis, France respectively. Despite their longevity and absence of more suitable alternative drugs, studies on the optimal use, variations in drug sensitivity, indications of resistance and mechanisms of action are still in progress.

Variation in the clinical response to the pentavalent antimonials, sodium stibogluconate, and meglumine antimoniate (Glucantime) in VL, CL and MCL has been a persistent problem in the treatment of leishmaniasis over the past 50 years. One explanation for this phenomenon is the intrinsic difference in sensitivity of the causative species to these drugs. In general, studies using the amastigote-macrophage model, L. donovani and L. braziliensis were shown to be three to five fold more sensitive to sodium stibogluconate than L. major, L. tropica and L. mexicana (Berman, 1981; Neal et al., 1995). Pentavalent antimonials are absorbed quickly and excreted rapidly from the body (half life of approximately 2 hours), whereas others take around 76 hours more (Chulay et al., 1988). Despite the differences between Leishmania species and their clinical presentation, the recommended treatment regimen for antimonials is fairly uniform.
### Table 1: Summary of characteristics of drugs in current use for Visceral leishmaniasis.

<table>
<thead>
<tr>
<th>Drug with Trade names</th>
<th>Regimen</th>
<th>Medication</th>
<th>Mechanism of action</th>
<th>Issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium stibogluconate (Pentostam®)</td>
<td>20 mg/kg daily for 20-40 days, depending on geographical area, intravenously</td>
<td>Cost (US$)</td>
<td>Antileishmanial activity might be due to action on host macrophages. Activated within the amastigote, but not in the promastigote by resistance. Quality in the promastigote by conversion to a lethal trivalent form.</td>
<td>Higher costs and toxicity when long course required because price of generic products</td>
</tr>
<tr>
<td>Amphoterin B(Fungizone)</td>
<td>7-20 mg/kg</td>
<td>60-150</td>
<td>Complexes with ergosterol in cell membrane, thus causing pores which alter ion balance and result in cell death appropriate; Need for intravenous infusion toxicity</td>
<td>Current second line treatment when</td>
</tr>
<tr>
<td>Liposomal amphoterin B(Ambisone)</td>
<td>10-20 mg/kg</td>
<td>1000-2500</td>
<td>Accumulated by the parasite; effects include binding to kinetoplast DNA. Primary mode of action uncertain. Alternative second line Treatment</td>
<td>Increasing unresponsiveness in</td>
</tr>
<tr>
<td>Pentamidine isethionate (Abelcet) C19H24N4O2</td>
<td>15-30 doses of 4 mg/kg over 3-4 weeks</td>
<td>60-150</td>
<td>In bacteria, paromomycin binds to 30S subunit ribosomes, causing misreading and premature termination of mRNA translation. In Leishmania, paromomycin also affects mitochondrial</td>
<td>Increasing nephrotoxicity and toxicity</td>
</tr>
<tr>
<td>Paromomycin sulfate (Hamatin) C23H47N5O2S</td>
<td>12-20 mg/kg for 20-30 days 10-20 per 21 day course</td>
<td>10-20 per 21</td>
<td>Inhibits protein synthesis by binding to 50S subunit ribosomes, causing misreading and premature termination of mRNA translation. In Leishmania, paromomycin also affects mitochondrial</td>
<td>Being an aminoglycoside, nephrotoxicity and</td>
</tr>
<tr>
<td>Miltefosine (Impavid) C21H46NO4P</td>
<td>50 mg for &lt;25 kg and 2.5 mg/kg for children for 28 days depending on dosage</td>
<td>50-100 per treatment</td>
<td>Possible inhibition of ether-remodeling, phosphatidylcholine biosynthesis, signal transduction and calcium homeostasis</td>
<td>Vomiting and diarrhea as a side effect. In high dose nephrotoxicity and hepatotoxicity</td>
</tr>
</tbody>
</table>
The patients are treated with 15-20 mg SbV/ kg/ day for 21-28 days and it is extended up to 40 days in resistance endemic regions either intramuscularly or intravenously (Berman, 1997). Treatment regimens differ depending on the species involved, health of the patients, healthcare facilities and infrastructure available to the clinicians. The long course of treatment allows accumulation of drugs to inhibitory levels in the liver and spleen tissues. The lengthy dose regimen of SbV treatment often causes side effects such as pancreatitis, cardiac arrhythmia and hepatitis leading to the reduction or cessation of treatment. Re-formulation of the drug reduce potential toxic side effects and increase activity of liposomal Pentostam against experimental CL and VL (Williams et al., 1998). However, there is no commercial impetus to reformulate this old drug.

After 50 years, the complex chemistry of meglumine antimoniate (Glucantime) has been characterized and a major moiety has been identified (Roberts et al., 1998). Concern about content was raised in 1995 by Franco et al, who showed that batches of Glucantime contained between 10 to 15% trivalent antimony. Trivalent antimony is more toxic to Leishmania and 10 -fold more toxic to humans than the pentavalent form (Roberts et al., 1995). Differential in vitro sensitivity of promastigotes and amastigotes to pentavalent antimonials in comparison to trivalent antimonials has been reported. It is still not clear whether this difference was due to influence of the macrophages. The development of systems to culture axenic amastigotes helped to clarify the situation to some extent. The results from axenic amastigote model confirmed that amastigotes have a greater intrinsic sensitivity to pentavalent antimonials than promastigotes.

7.0 Drug resistance - Current scenario

The threat of drug resistance is a major problem for all infectious diseases. In parasitic infections it has been best characterized for malaria (Talisuna et al., 2004). For leishmaniasis the situation is different. Although the selection of resistant lines of Leishmania has long been a part of laboratory studies on anti-leishmanial drugs, it is only in the past decade that acquired resistance has become a clinical threat, and this mainly is restricted to pentavalent antimonial drugs in one focus in India. Studies over the past decade have demonstrated an ever decreasing response rate of VL cases to antimonial treatment in Bihar, India.
Drug resistance is the reduction in effectiveness of a drug in curing a disease or improving a patient's symptoms. It has emerged as an impediment to the treatment and control of diseases of parasitic origin. The powerful emergence and global spread of antibiotic resistant microbial pathogens and resistance genes in the 1990s presents challenges to a wide range of disciplines from the molecular biology of resistance mechanisms, virulence genes and disease through molecular epidemiology all the way to drug design, infection control and medical practice.

Indeed, resistance of *Leishmania* against a given drug may be either natural or acquired when the parasites are exposed to suboptimal drug doses (Ouellette et al., 2004; Croft et al., 2006). The acquired drug resistance is expected in the region where anthroponotic transmission of leishmaniasis exists. Such drug resistance is becoming a problem in *L. infantum* and *L. donovani* mediated leishmaniasis, especially in the case of drug abusers infected with the parasite where transmission is from human to human by needle and by widespread misuse of the drug where medical practice is not controlled. However, the nature of the resistance cannot be generalized in the case of zoonotic transmission of cutaneous and visceral leishmaniasis caused by *Leishmania infantum* and *Leishmania chagasi* respectively.

Pentavalent antimonials were used worldwide for the treatment of leishmaniasis for more than 60 years without evidence of resistance. However, during the last 15 years increased clinical resistance has become concern particularly in patient groups co-infected with visceral leishmaniasis and HIV. Resistance against pentavalent antimonials represents one of the most serious problems in the control of Visceral leishmaniasis especially in area such as Bihar state of India (Grogl et al., 1992; Ouellette et al., 2004), where more than 70% of the cases do not respond to traditional antimonial therapy (Sundar et al., 2000; Croft et al., 2006).

Till date, the only reliable method for monitoring resistance in individual isolates is the use of technically demanding *in vitro* amastigote/macrophage (Croft et al., 2006) using parasite expressing reporter genes such as the firefly luciferase (Sereno et al., 2001) and recently described beta-lactamase assay (Mandal et al., 2009). Both of these assays require transfection of the parasites that does not modify the properties of the parasite.
related to macrophage infection and drug susceptibility (Gourbal et al., 2004; Ashutosh et al., 2005).

Currently, the drugs used in leishmaniasis treatment present several problems including high toxicity and many adverse effects, leading to withdrawal of patients from treatment and emergence of resistant strains. Biochemical mechanism of the drug resistance has been illustrated in Figure 4. The primary treatment against leishmaniasis includes pentavalent antimonials sodium stibogluconate and N-methylglucamine antimoniate forms, used since 1940 (Berman, 1988; Olliaro and Bryceson, 1993). In some cases, other drugs such as pentamidine, amphotericin B and paromomycin are used as a second option in resistant cases despite their great toxicity to the host (Ramos et al., 1990; Kuhlencord et al., 1992; Escobar et al., 2001; Bray et al., 2003). Recently, pentamidine resistant cases were also described in literature (Bray et al., 2003) along with difficulties in treatment of immune-depressed patients (i.e., HIV). Conventional drugs are less efficient and higher drug doses with prolonged treatment are normally required in these patients (Escobar et al., 2001).

7.1 Resistance to antimonial drugs

Treatment of VL has revolved around sodium stibogluconate for more than six decades. Small everyday doses for short duration (6-10 days) cured almost all patients till early eighties when reports of treatment failures started coming in, and modifications for SbV treatment were suggested to overcome the drug failure (Thakur, 1984). WHO revised its recommendations twice resulting in an increase in the daily dose (from 10 to 20 mg/kg) and duration (from 6-10 days to 20-40 days) (Report-WHO Expert Committee, 1984, 1990). In India there is no zoonotic reservoir, and only human to human (anthroponotic) transmission occurs, this means that once there is emergence of SbV refractory strains, their circulation in community occurs efficiently as SbV sensitive parasites gets eliminated by the drug and proportion of patients with SbV refractory parasites goes up very quickly. Thus it is apparent that SbV continues to be effective in the state of Uttar Pradesh, whereas in North Bihar, where most of the disease occurs, emergence of SbV unresponsive strains makes it ineffective in the vast majority of patients. In this major focus of VL, according to the World Health Organisation over 60% cases fail to respond to pentavalent antimonials at 6 month followup (Sundar et al., 2001).
The main question is whether this failure of response is due to acquired resistance, i.e. selection of resistant mutants. In one of the few reported studies it has been confirmed that resistance is a parasite rather than host factor (Lira et al., 1999).

Pentavalent antimonial drugs, pentostam and glucantime were used worldwide for the treatment of VL and CL for over six decades. Despite their extensive use, we remain uncertain of their mechanism of action, structures and even identities of the biologically active components of the optimal formulations (Ouellette et al., 2004). Unfortunately, the clinical value of antimony therapy is now challenged in several field sites (Ashutosh et al., 2005). VL endemic region in North Bihar and adjoining districts of Nepal have the unique distinction of being the only region in the world where widespread primary failure to Sb V has been reported (Thakur et al., 1998; Sundar et al., 2001). Even in this geographical region a variation in Sb V (van et al., 1985) sensitivity occurs with

![Figure 3: Biochemical mechanism of drug resistance](image-url)
significant drug resistance (Sundar et al., 2000). This resistance is so far unique to *L. donovani*; all isolates from a large number of refractory as well as responding patients in India were identified as this species (Sundar et al., 2001; Thakur et al., 2001).

### 7.2 Molecular mechanism of antimony resistance

After several decades of intensive research, the mechanism of resistance to antimonial drugs is nearly understood. Resistance to clinical drugs, the major impediment in the treatment of protozoal infection has always counted on the ease to develop drug resistant *in vitro* cell lines that has been instrumental in understanding the mechanism of drug resistance (Ouellette et al., 2004). There have been number of reports for generation of an *in vitro* sodium arsenite resistant cell lines in different *Leishmania* spp. to understand basic molecular mechanisms of drug resistance (Callahan and Beverley, 1991; Ouellette and Borst, 1991; Prasad et al., 2000).

The primary mechanism of resistance is the reduced active drug concentration within the parasite. This may be due to any of the following possibilities [a] Decrease in drug uptake [b] increased efflux [c] inhibition of drug activation and [d] alteration of the drug targets etc. The proposed model for antimony resistance in *L. donovani* is presented in (Figure 5).

### 7.3 Role of Aquaglyceroporins in antimony resistance

The route of entry of pentavalent antimonials (SbV) into *Leishmania* or into macrophages is not well understood although pentavalent form of arsenate (AsV) and antimony (SbV) are known to enter via a phosphate transporter (Rosen, 2002). In both prokaryotes and eukaryotes, aquaglyceroporins (AQPs) are known to transport trivalent metalloids (Sanders et al., 1997; Tsukaguchi et al., 1998; Tsukaguchi et al., 1999; Wysocki et al., 2001; Liu et al., 2002).

An aquaglyceroporin (AQP1) has also been identified and demonstrated to mediate the uptake of trivalent antimony in the *Leishmania* (Gourbal et al., 2004). Transfection of *AQP1* is also able to sensitize a SbV-resistant field isolate of *L. donovani* to sodium stibogluconate (SAG) due to increased accumulation of SbIII, thereby indicating its role in natural antimony resistance. Overexpression of *AQP1* also renders parasites hypersensitive to SbIII. These observations have been confirmed by a recent differential gene expression study in which the expression of *AQP1* was down-regulated at both the
promastigote and the intracellular amastigote stage in antimony-resistant clinical isolates from Nepal (Decuypere et al., 2005). The mRNA expressions of AQPI has also been shown to be low in antimony-resistant mutants of several Leishmania species (Marquis et al., 2005), thereby indicating its role in natural antimony resistance.

7.4 Inhibition of drug activation in antimony resistance

Recently, mass spectrometry (MS) approach has been used to demonstrate the accumulation of both SbV and SbIII form of antimony in both stages of the parasite (Brochu et al., 2003). Although SbV is accumulated in both stages of the parasite at pharmacological concentrations, it has no anti-leishmanial activity (Roberts and Rainey, 1993; Roberts et al., 1995; Sereno and Lemesre, 1997; Sereno et al., 1998).

Figure 4: Proposed mechanisms of antimony action and resistance in Leishmania spp (adapted from Croft et al., 2006). Levels of ornithine decarboxylase (ODC), γ-glutamylcysteine synthetase (γ-GCS), and an intracellular P-glycoprotein (PgpA) are elevated in some laboratory-derived resistant lines (thick lines), whereas decreased Sb reductase is observed in others. Dotted lines indicate nonenzymatic steps implicated in resistance. The red arrow indicates inhibition of trypanothione reductase and other targets. Uptake of Sb (III) is mediated via an aquaglyceroporin (AQP1).
However in some studies, axenic amastigotes have been found to be as sensitive to SbV as intracellular parasites (Callahan and Beverley, 1991; Ephros et al., 1999; Shaked-Mishan et al., 2001). The high anti-leishmanial activity of SbIII against both stages of *Leishmania* and the selective activity of SbV against the intracellular parasite support the hypothesis that the reduction of SbV to SbIII is necessary for activity.

As SbIII is highly active against both the stages of the parasite and SbV is active mostly against intracellular amastigotes, it is generally agreed that SbV needs to be reduced to SbIII. Reduction of metal takes place either in the macrophage (Sereno et al., 1998) or in the parasite (Shaked-Mishan et al., 2001) or in both generating higher lethal concentrations of SbIII within the parasite. Both the stages of the parasite can reduce SbV but amastigotes are more sensitive as compared to promastigotes (Shaked-Mishan et al., 2001). Even in pentostam resistant mutants, the ability to reduce SbV to SbIII is lost supporting the role of reducing activity in antimony resistance.

Non-enzymatic reduction of pentavalent to trivalent antimony due to reduced level of glutathione (GSH) and trypanothione (TSH) in the cells (Frezard et al., 2001; Ferreira et al., 2003; Yan et al., 2003) and enzymatic reduction of antimony in the presence of a parasite specific enzyme namely thiol-dependent reductase (TDR1) (Denton et al., 2004) has been explained. Although, TDR1 has been found to be highly abundant in the amastigote stage of the parasite, a direct relationship between the enzyme activity and antimony sensitivity in amastigote form of *Leishmania* species cannot be established.

Arsenate reductase (ScAcr2p) is ubiquitous in prokaryotes and eukaryotes and is essential for conferring resistance to arsenate (Mukhopadhyay and Rosen, 2002). Recently, the arsenate reductase homologue LmACR2 from *L. major* has been identified and characterized. The enzyme has been shown to catalyse the reduction of SbV thus increasing the sensitivity of *Leishmania* cells to SbV (Zhou et al., 2004). Most importantly transfection of LmACR2 in *L. infantum* promastigotes augments pentostam sensitivity in intracellular amastigotes confirming its physiological significance. It is also possible that more than one mechanism is responsible for drug activation.
### 7.5 Role of ABC-transporter (MRPA) in antimony resistance

Efflux of the drug is a very common resistance mechanism in bacteria, yeast and various pathogenic protozoa. The ABC transporter PGPA (renamed as MRPA) was found to be amplified in a number of laboratory mutants of *Leishmania* species selected for resistance to SbIII, SbV and AsIII (Callahan and Beverley, 1991; Ferreira-Pinto et al., 1996; Haimeur et al., 2000; Ouellette et al., 2001). Its role in antimony resistance was confirmed by transfection studies (Legare et al., 1997). However, this transporter is not responsible for the drug efflux across the plasma membrane. Rather, it confers resistance by sequestration of metal-thiol conjugates, a mode of metal detoxification in yeast cells (Legare et al., 2001; Rosen, 2002). MRPA is an intracellular transporter rather than an efflux transporter, thereby suggesting that MRPA may play a major role in antimony resistance (Weise et al., 2000). It is also over-expressed in the axenic amastigote stage of SbIII-resistant *L. infantum* (El et al., 2005). Recently mechanisms of antimony resistance in clinical isolates were demonstrated. Co-amplification of the pterin reductase gene (*ptrl*) and *pgpA* suggested amplification of the H-locus in the SAG resistant isolates.

### 7.6 Role of thiols in antimony resistance

Thiol metabolism has a central role in the maintenance of an intracellular reducing environment (Meister and Anderson, 1983). Antimony causes oxidative stress within the cell (Lecureur et al., 2002) which is lethal to the parasite. The presence of intracellular thiols helps to maintain intracellular reducing environment caused by SbIII oxidative stress. Trypanothione (TSH) was found to be increased in metal resistant *Leishmania* (Mukhopadhyay et al., 1996; Legare et al., 1997; Haimeur et al., 2000). The gene *gshl*, coding for γ-glutamylcysteine synthetase (γ-GCS), the rate limiting step in glutathione synthesis was also found to be amplified (Grondin et al., 1997; Haimeur et al., 2000) in antimony resistant *Leishmania*. In addition, the gene coding for ornithine decarboxylase (*ODC*), the rate limiting step in spermidine biosynthesis was also found to be overexpressed at the RNA level in AsIII resistant mutants (Haimeur et al., 1999). A dual increase in glutathione and spermidine levels, the two building blocks of trypanothione (TSH) leads to increase in TSH levels in drug resistant mutants. This suggests that lowering of intracellular thiol concentration may result in attenuation of resistant phenotype. This proposed hypothesis was confirmed by several inhibition studies.
(Grondin et al., 1997; Haimeur et al., 1999; Legare et al., 2001). Interestingly, SbV resistant *L. donovani* clinical isolates with significantly increased expression of γ-GCS was reversed in animal models by treatment with L-buthionine-sulphoximine (BSO), an inhibitor of γ-GCS (Carter et al., 2003; Carter et al., 2005). However in another study on *L. donovani* isolates from Nepal, expression of γ-GCS and ODC was significantly decreased in resistant isolates (Decuypere et al., 2005).

The role of tryparedoxin peroxidase (TryP) in Sb (III) resistance has been verified by overexpression of the recombinant *L. major* protein in Sb (III)-sensitive promastigotes (Wyllie et al. 2008). An approximate two fold increase in the level of TryP activity in this transgenic cell line was reported and a significant decrease in sensitivity to Sb (III) was observed. However, overexpression of an enzymatically inactive TryP failed to result in Sb (III) resistance. These results indicated that TryP-dependent resistance is not due to sequestration of Sb (III).

These resistant mechanisms derived mostly from studies on cells selected for resistance in the laboratory have yielded a coherent model for antimony resistance in *Leishmania* (Ouellette et al., 2004). The model has been supported by the studies carried out in Indian Visceral leishmaniasis clinical isolates (Mukherjee et al., 2007). Thus, natural antimony resistance mechanism in *L. donovani* is multifactorial with upregulation and downregulation of several genes.

8.0 Rationale of the present study

Visceral leishmaniasis (VL) is a parasitic disease caused by the protozoan parasite *Leishmania donovani*. Post-kala-azar dermal leishmaniasis (PKDL) is a cutaneous manifestation of visceral leishmaniasis (VL). Same parasite is considered as an agent for diverse clinical manifestations, such as visceral (VL) and dermal (PKDL) caused primarily by *L. donovani*. The contrasting immunological status of PKDL and VL patients has been reported. Basic underlying question that needs to be addressed is whether PKDL is a parasite determined manifestation of leishmaniasis. Cases of PKDL are of considerable epidemiological importance, acting as a reservoir of *L. donovani*. Therefore, the monitoring of antimonial resistance is a crucial issue in the anthroponotic focal region of leishmaniasis in India. The higher-dosage requirement for effective treatment of PKDL, as well as the increasing number of treatment failures in India, led us
to investigate the role that anthroponotic transmission plays in the spreading of drug-resistant *L. donovani* as a possible cause of unresponsiveness to SAG. Hence, the present research work is aimed to validate and identify the biomarkers associated with antimony resistance in the large number of VL and PKDL clinical isolates and also to identify global proteome differences between parasites isolated from VL and PKDL patients that may underlie the diversity in clinical manifestations of the disease.

9.0 Objectives of the present study

Towards molecular characterization of isolates from Visceral leishmaniasis and Post-kala-azar dermal leishmaniasis patients, thesis objectives are:

I. Development of an assay for the high-throughput screening of amastigote of *Leishmania donovani* clinical isolates against drugs using colorimetric β-lactamase assay.

II. Assessing aquaglyceroporin gene status and expression profile in antimony-susceptible and -resistant clinical isolates of *Leishmania donovani* from India.

III. Cloning, heterologous expression and characterization of aquaporins from *Leishmania donovani*.

IV. Screening of antimony -susceptible and -resistant isolates of *L. donovani* from Visceral and Post-kala-azar-dermal leishmaniasis patients for potential biomarkers.

V. Comparative proteome mapping of antimony -susceptible and -resistant clinical isolates of *L. donovani* using isobaric tag for relative and absolute quantitation (iTRAQ) method.

VI. Comparative proteome mapping of the clinical isolates of *L. donovani* from Post-kala-azar dermal leishmaniasis (PKDL) and Visceral leishmaniasis patients using quantitative Stable Isotope Labeling of Amino acids in Cell-culture (SILAC).