CHAPTER I

Introduction and Review of Literature
1.0 Overview

Leishmaniasis is a vector borne parasitic disease caused by 21 different species of protozoan parasites belonging to the genus *Leishmania* (Fig 1). It 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 are in the new world. The disease is transmitted to humans by the bite of more than 30 species of female sandfly vector *Phlebotomine* spp. in the old world (Shaw, 1994). The new world leishmaniasis vector is *Lutzomyia langipalpis*. Human leishmaniasis consists of mainly two clinical forms, simple self healing cutaneous leishmaniasis (CL) and; disfiguring and debilitating or even fatal, the visceral leishmaniasis (VL). Visceral leishmaniasis is also known as kala-azar. 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 (LR) and post-kala-azar dermal leishmaniasis (PKDL) that are often associated with the host immune status.

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. 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. Hence, there is an urgent need to understand the drug resistance mechanism in the field isolates.

This thesis deals with molecular characterization and validation of biomarkers of antimony resistance in the *Leishmania donovani* field isolates.
Figure 1: Taxonomy of *Leishmania*. At present, 21 species of *Leishmania* are known to be pathogenic for humans, belongs to 9 *Leishmania* complex. The classification is based on the Scheme published by the World Health Organization (WHO, 1990) with addition from literature (adopted from Banuls et al., 2007. Advances in parasitology Vol. 64)
2.0 Present status of disease

2.1 Global distribution

World wide, 12 million humans are estimated to be infected with an incidence of 0.5 million cases of the visceral form of the disease and 1.5 to 2.0 million cases of the cutaneous form of the disease. 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 (Fig 2). Currently, the main foci of visceral leishmaniasis (VL) are in Sudan and India and those of cutaneous leishmaniasis (CL) are in Afghanistan, Syria and Brazil.

Visceral leishmaniasis is present in 70 countries. The largest focus of VL is in the South-east Asian region followed by East Africa and America. New foci are appearing at an alarming rate and incidence in East Africa is on the increasing trend. Annually 90% of VL occurs only in five 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. Several clinical manifestations of CL have been reported: localized CL which often heals without treatment, diffused CL which is very difficult to treat and mucosal leishmaniasis which is the most severe form producing disfiguring lesions and mutilation of the face. Ninety percent of all mucocutaneous leishmaniasis (MCL) occur in Bolivia, Brazil and Peru.

2.2 Leishmaniasis: Indian sub-continent

The Indian sub-continent - India, Bangladesh and Nepal account about 400,000 new cases of leishmaniasis every year (Joshi A, 2008) (Fig 3). 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: 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. It is caused by Leishmania donovani. The disease first appeared as an epidemic form in India in 1857 affecting people of the Hoogly district of West Bengal and subsequently in 1962 in district Burdwan (The Burdwan fever). Currently, leishmaniasis is endemic in four states of India
Figure 2: Global distribution of leishmaniasis
(http://www.who.int/emc/disease/leish/leisgeo1.html)
Figure 3: VL endemic areas in Bangladesh, India and Nepal. (Adopted from Joshi A.B. et al., 2008)
Introduction and Review of Literature

(Bihar, Jharkhasnd, Uttar Pradesh and West Bengal). Bihar accounts for more than 90% of the total cases, where daily family income is approximately US$ 1 (Sundar et al., 1998; Sundar and Rai, 2002; Thakur et al., 2000b). The 30 districts of Bihar, 8 districts of West Bengal and 2 districts of Uttar Pradesh are the highly endemic districts in India 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.

Nepal: In Nepal, eastern Terai region of the country, adjacent to the Indian state of Bihar is mostly affected with visceral leishmaniasis. VL in Nepal was reported to be endemic in Nepali lowlands in 1950's but no formal surveillance existed at that time. The first confirmed case of VL was recorded in 1980. Since then, the central and eastern lowland region of the country have experienced resurgence of VL in parallel with large epidemic in the neighbouring Indian State of Bihar, Uttar Pradesh and West Bengal (Herwaldt, 1999; WHO, 1990). Currently, the disease is endemic in 14 districts of the country where nearly six million people residing in these districts are at the risk of acquiring the disease. Along with the visceral leishmaniasis, PKDL (Karki et al., 2003) and CL (Pandey et al., 2006) cases have also been reported in Nepal.

Bangladesh: In Bangladesh, sporadic kala-azar cases were reported in the 1970s and outbreak occurred in Pabna district in 1980 (Elias M et al., 1989). The districts most affected in the early 1980s were reported to have been Sirajganj, Pabna, Mymensingh, Rajshahi and Tangail (Elias M et al., 1989). At present, 34 out of 64 districts of Bangladesh are highly affected with visceral leishmaniasis (Bern and Chowdhury, 2006).

3.0 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 macrophages 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 (Fig 4). 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 hemocoel. 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.

Most leishmaniasis are zoonotic form (animal reservoir host), where humans becomes infected only when accidentally exposed to the infective sandflies. However, humans are probably the sole reservoir host in the anthroponotic form. The reservoir of *L. infantum* and *L. chagasi* is usually dog where as reservoir of *L. donovani* is mainly human (Hommel, 1999). Domestic dog is incriminated as a reservoir host of American cutaneous leishmaniasis caused by *L. braziliensis, L. panamensis* and *L. peruviana* (Reithinger and Davies, 1999). Gerbils (*Psammomys obesus*) have been implicated as a reservoir host of *L. major* in different Asian countries (el-Sibae and Eesa, 1993; Elbihari and el-Hassan, 1987; Rioux et al., 1990).

### 4.0 Clinical features

Clinical spectrum of leishmaniasis is shown in (Fig 5).

#### 4.1 Visceral leishmaniasis (VL)

Visceral leishmaniasis is distributed in South and Central America, Africa, Mediterranean region, Indian sub-continent and China. It is the most severe form of the disease. The parasite invades internal organs (spleen, liver, bone marrow) and if left
Figure 4: Leishmanial life cycle in mammalian host and sand fly vector.

- Delivery of promastigotes into human skin by the bite of sand fly vector.
- Engulfment by phagocytosis of promastigotes by macrophages
- Differentiation of promastigotes into amastigote in the phagolysosome of the infected macrophage.
- Multiplication of amastigotes and rupture of heavily parasitized macrophage and release of amastigotes.
- Ingestion of parasitized macrophage by sand fly after blood meal taken from an infected person or reservoir animal.
- Ingestion of parasitized cell and replication of amastigotes
- Differentiation of amastigotes into promastigotes in the mid gut of the sand fly.
- Replication of promastigotes in the abdominal mid gut and its migration to the pharynx and buccal cavity. Actively motile promastigotes (metacyclic form) are found in the proboscis or mouth part of the sand fly.
Figure 5: Pathology of leishmaniasis.

A. Visceral leishmaniasis patients. Note: enlarged liver and spleen.

B. A patient with diffused anergic cutaneous leishmaniasis.

C. A patient with post Kala-azar dermal leishmaniasis.

D: Patients with mucocutaneous leishmaniasis showing deformed nose, lips etc
untreated may lead to mortality. The infection follows reticuloendothelial hyperplasia affecting spleen, liver, mucosa of small intestine, bone marrow, lymph nodes and other lymphoid tissue. The life span of leukocytes and erythrocytes is reduced, causing anemia. Prothrombin production is reduced, causing mucosal hemorrhage. Other symptoms include fever, diarrhoea, weight loss and loss of appetite.

VL is caused by *L. donovani* in the Indian subcontinent and in East Africa. In the Mediterranean region, it is caused by *L. infantum*. In the New World mainly in Brazil, Peru and Paraguay the disease is caused by *L. chagasi* (Berman, 1997) which is closely related to *L. infantum* (Mauricio et al., 2000).

Visceral leishmaniasis co-infection with human immunodeficiency virus (HIV) cases is also increasing now a day probably due to human migration and resettlement. The association of VL and HIV infection clearly confirms the fact that VL is an opportunistic infection. HIV/Leishmania co-infections are considered to be a real threat; especially in Southern Europe where approximately 700 cases of co-infection have been reported to World Health Organization (WHO). Acquired Immunodeficiency Syndromes (AIDS) and VL are locked in a vicious circle of mutual reinforcement. VL accelerates the onset of full-blown AIDS and shortens the life expectancy of HIV-infected people while HIV spurs the spread of VL. The gridlock produces cumulative deficiency of the immunoresponse as Leishmania parasites and HIV destroy the same cells (WHO, 1999).

4.2 Post kala-azar dermal leishmaniasis (PKDL)

PKDL is a dermatropic form of leishmaniasis developed by part of the treated VL patients (WHO, 1990) in a year’s time (Napier LE and Das Gupta RC, 1981) but there are cases without any 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 (Ramesh V and Mukherjee A, 1995; Zijlstra et al., 1994; 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 V and Mukherjee A, 1995; Zijlstra et al., 2003).

4.3 Cutaneous leishmaniasis (CL)

CL is also known as oriental sore. It produces skin lesions on the face, arms and legs, causing serious disability and permanent scars (WHO, 1999). Infection by parasites of the *L. major*, *L. tropica* and *L. aethiopica* complexes of species (Old World) and of the *L. mexicana* and *L. braziliensis* complexes (New World) usually gives rise to CL. Some *L. infantum* and *L. donovani* strains can also cause lesions. Ninety percent of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually world-wide (WHO, 1999). CL caused by *L. major* is usually more chronic and is the most severe form. In the New World, *L. Mexicana* usually produces relatively benign lesions (Desjeux, 1996).

4.4 Mucocutaneous leishmaniasis (MCL)

Espundia or mucocutaneous leishmaniasis is caused by *L. Mexicana*, *L. braziliensis* and *L. guyanensis*. In this nonhealing leishmaniasis, the initial skin lesion may cure but metastatic lesions develop in the mucosa of nasopharynx. Thus the parasite affects the mucocutaneous tissue of the nose, mouth and throat cavities and surrounding tissues and leads to gross disfigurement in these areas, mutilation of the face and great suffering for life (Desjeux, 1996; WHO, 1999). Ninety percent of all cases of MCL occur in Bolivia, Brazil and Peru (WHO, 1996).

4.5 Diffuse cutaneous leishmaniasis (DCL)

It is less common, chronic in evolution and especially difficult to treat. It produces disseminated and chronic skin lesions resembling those of leprotamous leprosy, which do not heal spontaneously due to the deficiency of immune response (Desjeux, 1996; WHO, 1999). DCL is caused by *L. aethipica* and *L. amazonensis* (Desjeux, 1996).
5.0 Current Status of Chemotherapy

Historically, the earliest kala-azar epidemic occurred in 1824 in Jessore district of India (now in Bangladesh) which killed several thousands of people. Potassium antimony tartrate was used to treat visceral leishmaniasis for the first time (Steck, 1974). However, the drug was more toxic and produced many side effects. Later on, in the year 1912 pentavalent antimonial compound stibamine (SbV) was discovered. In 1922, Dr. Brahmachari showed that the stibamine compound (SbV) can be effectively used against visceral leishmaniasis (Singh and Sivakumar, 2004). Since then, it is used as the drug of choice in the treatment of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) in many countries of the world.

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 (Table 1) (Croft and Coombs, 2003; Croft and Yardley, 2002). The chemical structures of the commonly used drugs are given in Fig. 6. 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., 2000a) 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 (Asilian et al., 2003; el-On et al., 1992; Soto et al., 2002).

Cost issues prevent the use of liposomal drugs in most countries where the mainstay of treatment is still prolonged intravenous treatment with antimonials, despite ever-increasing patterns of resistance and an increasing incidence of treatment failures. In case of India and adjoining districts of Nepal where resistance towards pentavalent...
Table 1. Current drugs for leishmaniasis
(parenteral administration unless otherwise stated)

<table>
<thead>
<tr>
<th>Type of leishmaniasis</th>
<th>Status of drugs</th>
<th>Drugs</th>
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<tbody>
<tr>
<td>Visceral</td>
<td>First line drugs</td>
<td>Sodium stibogluconate; meglumine antimoniate</td>
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<td></td>
<td></td>
<td>Amphotericin B (Fungizone)</td>
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<td></td>
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<td>Liposomal amphotericin B (AmBisome)</td>
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<td>Pentamidine</td>
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<td>Clinical trials</td>
<td>Miltefosine (oral, phase IV)</td>
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<td></td>
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<td>Paromomycin (phase III)</td>
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<td>Stimaquine (oral, phase II)</td>
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<td></td>
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<td>Other amphotericin B formulations</td>
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<td>Cutaneous</td>
<td>First line drugs</td>
<td>Sodium stibogluconate; meglumine antimoniate</td>
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<td></td>
<td></td>
<td>Amphotericin B (Fungizone)</td>
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<td></td>
<td>Pentamidine</td>
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<td></td>
<td></td>
<td>Paromomycin (topical formulations with methylbenzethonium chloride or ures)</td>
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<tr>
<td></td>
<td>Clinical trials</td>
<td>Miltefosine (oral, phase III; registered in Columbia in 2005)</td>
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<tr>
<td></td>
<td></td>
<td>Paromomycin (topical formulation with gentamycin and surfactants, phase II)</td>
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<td></td>
<td></td>
<td>Imiquimod (topical immunomodulator, phase II)</td>
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<tr>
<td></td>
<td></td>
<td>Antifungal azoles (ketoconazole, fluconazole, itraconazole)</td>
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</tbody>
</table>
Figure 6: Chemical structures of antileishmanial drugs.

http://parasites.trends.com
antimonials are endemic, alternative treatment such as amphotericin B is used. In terms of oral medications, miltefosine is the sole agent that has been shown to be effective. Miltefosine is currently approved in India for visceral leishmaniasis.

5.1 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. brasiliensis* 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. 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 in both orders of magnitude 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.

### 5.2 Amphotericin B

Amphotericin B is used as a second-line drug in the treatment of mucocutaneous and visceral leishmaniasis after the failure of antimonial therapy. It has been recommended as a first line drug by the Indian National Expert Committee for pentostam resistance regions. It is a macrolide polyene antibiotic isolated from *Streptomyces nodosus* (Fig.3) and was first shown to have anti-leishmanial activity in the early 1960’s. Amphotericin B is predominantly used as an antifungal drug specifically for the treatment of systemic mycoses. The selective activity of amphotericin B against fungi and *Leishmania* is due to the higher affinity of the drug against 24-substituted sterols found in the plasma membrane of these eukaryotic microorganisms over cholesterol in the plasma membranes of mammalian cells. It is also a toxic drug and its adverse reactions include — universal occurrence of infution-related fever, chills, vomiting and diarrhea, high incidence of thrombophlebitis and occasionally fatal hypokalemia, renal impairment and cardiac toxicity. In particular, studies have shown higher cure rates of VL including SbV resistant cases in India even at doses reduced from 1 mg/ kg/ day to 0.5 mg/ kg/ day for 15 days in case of children (Mishra et al., 1994; Thakur et al., 1999).
The potential of liposomal amphotericin B was reported to be better in activity than none-liposomal form. New lipid formulation of Amphotericin B (AmBisome) is taken up well by the reticuloendothelial system and poorly by the kidney. These formulations target the cells that host the parasite and have decreased nephrotoxicity. The major limitation is its high cost which remains beyond the budgets of most endemic countries.

5.3 Pentamidine

Pentacarinat, isothionate salt of pentamidine (aromatic diamidine) and lomidine (methylsulphonate salt) have been used as alternative treatments for both VL and CL since 1952 and as a primary treatment for *L. aethiopica* which causes DCL. It has been proved to be useful as a second line drug for antimony resistant cases in India and Kenya (Thakur et al., 1991). However, the declining efficacy of the drug coupled with serious adverse effects like hypoglycemia, shock and occasional death in significant proportion of patients leading to almost abandonment of the drug (Jha et al., 1991; Mishra et al., 1992; Thakur et al., 1991). Pentamidine was shown to be highly effective against CL in Colombia in a short course low dose regimen (Soto et al., 1994).

5.4 Paromomycin

Paromomycin (PM), an aminoglycoside antibiotic (Fig 5) was originally identified as an antileishmanial in the 1960s and has been used in clinical trials for both VL and CL. Although development of the parenteral formulation of PM, a drug with poor oral bioavailability, has been slow, several Phase III trials in India and Kenya have been promising, with 90% of patients cured of VL following treatment with 15 mg/ kg daily for 20 days, including antimony-refractory cases (Thakur et al., 2000a) and now it is on phase IV clinical trials in India. There are also encouraging findings on the use of PM as a topical treatment for CL.

5.5 Miltefosine

The most significant recent advance has been the effective oral treatment of VL by using miltefosine or hexadecylphosphocholine an alkyl phosphocholine (Fig 5) originally developed as an anticancer drug (Croft and Coombs, 2003). The activity of
miltefosine was compared to that of another phospholipid analogue, also initially developed as an anticancer drug, edelfosine (ET-16-OCH3). The antileishmanial activity of miltefosine was initially discovered in the mid-1980s, and the subsequent demonstration of its efficacy in several experimental models (Croft et al., 2003) led in the mid-1990s to clinical trials and co-development of miltefosine for leishmaniasis by a partnership between Asta Medica (now Zentaris) and WHO/TDR. After a Phase III trial, in which 282 out of 299 (94%) VL patients were cured with an oral dose of 2.5 mg/ kg of miltefosine daily for 28 days (Sundar et al., 2002); miltefosine was registered in India in March 2002 for oral treatment of VL. Over 700 patients including many who were refractory to antimonials have now been successfully treated (Sundar et al., 2002). Miltefosine has also proved to be active against CL in a clinical trial in Colombia (Soto et al., 2001) and further trials against this disease are planned. Mechanism of miltefosine is likely to be due to inhibition of phospholipid and sterol biosynthesis via interference with cell signal transduction pathways. The major limitation of miltefosine is teratogenicity and this excludes its use in women of child-bearing age.

5.6 Azoles

Azoles offer potential of “therapeutic switching” or “piggy-back” chemotherapy. They were developed as antifungal drugs. Leishmania resemble fungi in synthesizing 24-substituted sterols such as ergosterol, whereas mammals have just cholesterol. Azoles, such as ketoconazole, inhibit 14α-demethylase, a key enzyme in this sterol biosynthesis pathway. Ketoconazole, itraconazole and fluconazole have undergone several trials for CL and VL with equivocal results. In one controlled trial, ketoconazole was found to have some activity against \(L.\) mexicana, but not against \(L.\) braziliensis infections (Navin et al., 1992). Recently, the oral activity of posoconazole in a \(Leishmania\) amazonensis in experimental model has proved to be encouraging (Al-Abdely et al., 1999).

5.7 Sitamaquine

Sitamaquine, a 4-methyl-6-methoxy-8-aminoquinoline (lepidine), previously known as WR6026, is in phase II(b) clinical trials by GlaxoSmithKline for the treatment of VL. The drug has broad-spectrum of anti-protozoal activity (Yeates, 2002) but with
limited clinical use and no reported resistance. In addition, there is no literature on sensitivity of *Leishmania* species.

Sitamaquine was found to be 200 times more active than primaquine against *L. donovani* in hamsters *in vivo* but only twice as active as primaquine *in vitro* (Kinnimon et al., 1978). Like primaquine, this compound appears to undergo hydroxylation and N-alkylation by rat hepatic microsomes (Theoharides et al., 1985). The activity of sitamaquine metabolites against *Leishmania* spp. has not been reported. The mode of action is not known but could involve "futile redox cycling" as proposed for primaquine.

### 5.8 Nucleoside Analogs

In the 1980s, allopurinol, a pyrazolopyrimidine, entered clinical trials for the treatment of VL and CL alone and in combination with antimonials (Croft and Yardley, 2002). Although not a successful treatment for human disease, it is still used in the treatment of canine leishmaniasis (Koutinas et al., 2001). Allopurinol is known to inhibit enzymes of the purine salvage pathway in *Leishmania* (Nelson et al., 1979). In comparative studies wide variations in sensitivity of the promastigotes of different species to the pyrazolopyrimidines allopurinol and allopurinol riboside were reported to be due to differences in the affinity of enzymes of the purine salvage pathway (Nelson et al., 1979).

### 6.0 Drug resistance - Current scenario

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 (Croft et al., 2006;
Ouellette et al., 2004). The acquired drug resistance is expected in the region where anthropoponotic 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 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 (Croft et al., 2006; Cruz et al., 2002; Sundar et al., 2000). Till date, the only reliable method for monitoring resistance of 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 (Ashutosh et al., 2005; Gourbal et al., 2004).

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 Fig. 7. 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
Figure 7: Biochemical mechanism of drug resistance [Adapted from Hayes et al. Biochemical J. (1990), 272, 281-295]
the host (Bray et al., 2003; Escobar et al., 2001; Kuhlencord et al., 1992; Ramos et al., 1990). 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).

6.1 Resistance to antimonial drugs

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 (Sundar, 2001; Thakur et al., 1998). Even in this geographical region a variation in Sb(V) sensitivity occurs with 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, 2001; Thakur et al., 2001).

6.2 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; Detke et al., 1989; Ouellette et al., 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: (i) Decrease in
drug uptake (ii) increased efflux (iii) inhibition of drug activation and (iv) alteration of the drug targets etc. The proposed model for antimony resistance in *L. donovani* is presented in (Fig. 8).

6.3 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 (AQP5) are known to transport trivalent metalloids (Liu et al., 2002; Sanders et al., 1997; Tsukaguchi et al., 1998; Tsukaguchi et al., 1999; Wysocki et al., 2001). An aquaglyceroporin (AQPI) 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 *AQP1* 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.

6.4 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 et al., 1995; Roberts and Rainey, 1993; Sereno et al., 1998; Sereno and Lemesre, 1997). However in some studies, axenic amastigotes have been found to be as sensitive to SbV as intracellular parasites (Callahan et al., 1997; Ephros et al., 1999; Shaked-Mishan et al., 2001). The high antileishmanial activity of SbIII against both stages of *Leishmania* and
Figure 8: Proposed mechanisms of antimony action and resistance in Leishmania spp. Levels of ornithine decarboxylase (ODC), -glutamylcysteine synthetase (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).
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 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 (Ferreira et al., 2003; Frezard et al., 2001; 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 Leishmania 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 Leishmania 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.

6.5 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). MRP A is an intracellular transporter rather than an efflux transporter, thereby suggesting that MRP A may play a major role in antimony resistance (Weise et al., 2000). It is also overexpressed 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 (*PTR*1) and MRP A suggested amplification of the H-locus in the SAG resistant isolates.

### 6.6 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* (Haimeur et al., 2000; Legare et al., 1997; Mukhopadhyay et al., 1996). The gene *GSH1*, coding for γ-glutamylcysteine synthase (*γ-GCS*), the rate limiting step in GSH 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 GSH and spermidine levels, the two building blocks of 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 resistance *L. donovani* clinical isolates with significantly increased expression of *γ-GCS* was reversed in animal
Figure 5. PCR-RFLP of *HSP70* gene of the *L. donovani* clinical isolates. PCR-RFLP banding pattern of *HSP70* gene of *L. donovani* isolates after digestion with *HaeIII* enzyme on 1.5% agarose gel.
7.1 Functional cloning

Functional cloning is a whole genome approach ideally suited for the isolation of drug resistance genes (Ouellette et al., 2008). This approach is applicable in case where resistance phenotype is due to either gain of a function or loss of a function. The strategy includes, preparation of a genebank in an expression cosmid-vector (Kelly et al., 1994; Ryan et al., 1993) followed by transfection in organisms (either in wild type or resistant one depending upon the nature of the study) and finally drug susceptibility assays. If it is hypothesized that the resistance is due to over-expression of a target gene i.e. gain of the function, cosmid bank is transfected into the wild type organism. The transfected cells are selected by clonal selection method with the cosmid selection marker (e.g hygromycin, neomycin etc). Individual clones are picked up and grown in microtitre plate with cosmid selection marker drugs alone or both selection marker drug and various concentration of the drug to be tested. This strategy of functional cloning is called dominant positive approach. It has been used for testing several drugs in Leishmania such as heat shock protein 70 (HSP70) gene with antimonial drugs (Brochu et al., 2004); ABC transporter (PRP1) gene with Pentamidine (Coelho et al., 2003); Bioppterin transporter (BTL), Pteridine reductase (PTR1) and dihydrofolate reductase (DHFR) genes with Methotrexate (Cotrim et al., 1999) etc. If it is hypothesized that the resistance is due to the down-regulation of the target gene i.e loss of function, cosmid bank is transfected into the resistant organism and selected with the cosmid selection marker drug. This approach is called dominant negative approach. P-type phospholipid translocase (LDMT) gene (Perez-Victoria et al., 2003) and Aquaglyceroporin (AQP1) gene in relation to miltefosine and antimony resistance (Marquis et al., 2005) were identified using this dominant negative approach of functional cloning.

7.2 DNA micro-array

DNA microarray is a powerful tool used to detect differential expression of gene in Leishmania among the several other techniques (Charest et al., 1996; Lamontagne and Papadopoulou, 1999; Quijada et al., 2005; Wu et al., 2000). The technique was used to monitor gene expression in Candida albicans selected for resistance to fluconazole or amphotericin B (Barker et al., 2004; Cowen et al., 2002) in Leishmania cells made
resistant to different classes of drugs \textit{in vitro} (El et al., 2005; Guimond et al., 2003; Leprohon et al., 2006) and in number of bacteria species (Haas et al., 2005; Mongodin et al., 2003). Recently Ouellette and his group generated 70-mer oligonucleotide full genome arrays of \textit{Leishmania} which is expected to be useful for analyzing resistance at the global RNA expression level. This has the advantage of detecting several changes of gene expression at once on the whole genome level in a fairly comprehensive fashion. Obviously, changes in RNA expression are not always correlated to changes in protein levels (McNicoll et al., 2006) but in case of resistance this may differ as several differentially expressed RNAs have been linked to resistance (Ouellette et al., 2008).

\textbf{7.3 Proteomics}

Proteomics is another powerful technique recently developed for studying antimicrobial resistance (Papadopoulou et al., 2004). One advantage of proteomics over DNA microarrays is that proteins are closer to function than RNA. Proteomics also allows the detection of post-translational modifications (PTM) (Drummelsmith et al., 2003). Another advantage of proteomic techniques is the possibility of obtaining clues to function by cellular localization (Foucher et al., 2006). Proteomic techniques have proved useful to find an alkyl hydroxyperoside reductase possibly involved in ergosterol biosynthesis in azole-resistant \textit{C. albicans} (Hooshdaran et al., 2004) and have also found over-expression of a phosphate ABC transporter protein in penicillin-resistant \textit{Streptococcus pneumonia} (Soualhine et al., 2005). In \textit{Leishmania}, the technique has been used to demonstrate overproduction of the pterin reductase (PTR1) (Drummelsmith et al., 2003) and of the methionine adenosyl transferase (MAT) (Drummelsmith et al., 2004) in MTX-resistant parasite.

\textbf{7.4 Point mutations}

Point mutations are often linked to drug resistance. Recently, NimbleGen (www.nimblegen.com) has developed a technique using overlapping small oligonucleotides for the detection of point mutations and other polymorphisms in bacterial genomes and this technique has been used successfully in the context of resistant organisms (Albert et al., 2005). The method consists resequencing of whole
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genomes of organisms using hybridization based microarrays for the detection of point mutations (Ouellette et al., 2008). Proteomic approach for detection of point mutation includes differential migration in 2D gel.

At present, several genes associated with antimony resistance have been identified in Leishmania laboratory mutants using either whole genome approach or other methods. However, their relevance in natural antimony resistance needs to be validated. Hence, validation of their role will help to develop biomarkers for monitoring antimony resistance cases in the field. That will help to choose better treatment option as well as future drug discovery to reverse the resistance.

8.0 Aquaporins and aquaglyceroporins

Aquaporins (AQPs) are members of a large family of integral membrane proteins which mainly facilitate water transport across cell membranes (Agre and Kozono, 2003; Casotti et al., 2007; Giffard-Mena et al., 2007; Suzuki et al., 2008). The aquaporin family is differentiated into two groups according to their functionality: orthodox aquaporins (AQP) and aquaglyceroporins (GLP). The aquaporins facilitate transport of water while aquaglyceroporins transport small non-ionic solutes such as glycerol and urea. Till date, 13 members of the AQP family (AQP0–AQP12) have been identified in human (Borgnia et al., 1999). Among them AQP3, 7, 9 and 10 are classified as aquaglyceroporins (Agre and Nielsen, 1996; Magni et al., 2006; Verkman, 2002). Leishmania genome sequencing project has identified five aquaporin homologous families. Aquaglyceroporin (AQPI) is the only gene characterized till date among them.

8.1 Molecular feature

The quaternary structure of the protein is a homotetramer. Each major intrinsic protein (MIP) monomer contains six hydrophobic membrane-spanning helices (1-6) connected by five loops (A-E) that delimit a polar channel with two wide periplasmic vestibules and one central pore (hourglass model). The loops B (cytoplasmic) and E (extracellular) interact with each other from opposite sides through two highly conserved NPA (Asn-Pro-Ala) boxes forming one of the narrowest region of the pore. A single N-glycosylation site present in the extracellular loop C and a cysteine residue in loop E is
known to be responsible for mercurial sensitivity of most MIPs (Preston et al., 1993). The most conserved feature of the protein is the presence of NPA motif in loops B and E (Fig 9 A and B).

8.2 Permeability mechanism

Water selectivity of orthodox aquaporin (AQP1) is basically due to a simple size exclusion effect. AQP1 has a constriction of about 2.8 Å in diameter in the aromatic/R region. It represents the size of water so that solutes larger than a water molecule can not pass the AQP1 pore (Murata et al., 2000; Unger, 2000). According to the proposed model of Murata et al., (2000), hydrogen interchange can be inhibited by breaking the hydrogen bonding pattern along a single file column of about seven water molecules. The two conserved N of the NPA motifs stabilize the central water aligned perpendicular to the channel axis. This conformation converts the central water molecule to a hydrogen bond donor to its neighbouring water molecules. Therefore, an organization of two opposite water molecules orientation in the two halves of the channel occurs. This bi-orientational ordering of the water- wire prevents the contiguous hydrogen-bonded water chains through the channel, a necessary condition for proton conductance (de Groot et al., 2001; Murata et al., 2000; Unger, 2000).

The selectivity in glycerol facilitator GlpF depends on the spatial structure of its pore. The diameter of the channel opening measures about 3.5 Å at its narrowest point and the constriction region of the channel is approximately 28 Å long. Due to this geometric constraint, the substrates can only pass in single file, exactly three glycerol molecules at a time (Fu et al., 2000). The hydrophobic backbone of glycerol is oriented towards the apolar residues. It is particularly tightly packed against two hydrophobic amino acids.

8.3 Aquaglyceroporin as metalloid transporter

Aquaglyceroporin (GlpF) is the first identified glycerol transporter in E. coli. It is a member of the major intrinsic protein (MIP) superfamily that allows the transport of water and small solutes such as glycerol and urea by an energy-independent mechanism. Aquaglyceroporin (GlpF) has been shown to facilitate the adventitious uptake of AsIII
Figure 9: Molecular features of *Leishmania donovani*, aquaglyceroporin (AQP1) protein. (A) Topology prediction of aquaglyceroporin transmembrane protein was carried out using the HMMTOP online software (http://www.enzim.hu/hmmtop/) version 2. Six helical (1-6) transmembrane segments and topology of the *L. donovani* aquaglyceroporin protein was identified and constructed the protein map. A, C and E are extracellular loops, B and D are intra cellular loop. NPA motifs are in transmembrane region. (B) The hour glass model of *L. donovani* AQP1 protein. The six transmembrane domains (1-6) are connected by five loops (A-E) and delimit a central pore conserved NPA boxes interact with each other at the narrowest region of the channel.
and SbIII. In *Sinorhizobium meliloti* (prokaryotes) and *Saccharomyces cerevisiae* (Eukaryotes), GlpF homologous aquaglyceroporin (*FpsI*) gene has been identified and has been shown to play a role in AsIII and SbIII transport (Liu et al., 2002; Wysocki et al., 2001). Similarly, aquaglyceroporin family such as hAQP3, hAQP7, hAQP9 and hAQP10 has been shown to transport AsIII in human (Liu et al., 2004).

*Leishmania major* genome consists of five aquaporin homologues genes: *LmAQP1*, *LmAQPα*, *LmAQPβ*, *LmAQPγ* and *LmAQPδ*. *LmAQP1* is more similar to bacterial AQP5 while the other aquaporins (*LmAQPα - δ*) are closer to plant AQP5s. This is a peculiarity of aquaporins of *Leishmania* because other parasitic AQP5s known till date are either bacteria- or plant-like not a mixed type (Beitz, 2005). *LmAQP1* is localized exclusively to the flagellum of promastigotes. It is found in the flagellar pocket, rudimentary flagellum and contractile vacuoles of the amastigotes (Figarella et al., 2007). *LmAQP1* plays an important physiological role in water and solute transport, volume regulation and osmotaxis.

The role of aquaglyceroporin (*AQP1*) gene in AsIII and SbIII transport has been shown in *Leishmania* by transfection of *AQP1* into three different species of *Leishmania* (*L. tarentolae*, *L. infantum* and *L. major*) (Gourbal et al., 2004). Overexpression of *AQP1* in laboratory induced resistant parasite restored sensitivity, independent of the mechanism of resistance. Correlation between increased uptake of SbIII and hypersensitivity of the *L. donovani* promastigotes has been shown (Maharjan et al., 2008) in field isolates. Gene knockout studies have shown that disruption of one allele of *LmAQP1* results in a ten fold increase in resistance to trivalent antimony compared to the wild type (Gourbal et al., 2004). Earlier reports indicated that loss of *AQP1* can produce resistance while increased expression can reverse resistance. *AQP1* mRNA was also shown to be decrease significantly in experimentally induced SbIII or AsIII resistant *L. major* and *L. tarentolae*. (Marquis et al., 2005). Similarly, pentostam-resistant field isolates of *L. donovani* from Nepal showed downregulation of *AQP1* and reduced antimony uptake (Decuypere et al., 2005). These evidences indicated that aquaglyceroporin gene has an important role in development of antimonial resistance in leishmaniasis.
9.0 Heat Shock Protein (HSP70)

Heat shock proteins (HSPs) are highly conserved proteins found in both prokaryotic and eukaryotic cells. The protein was reported for the first time in *Drosophila* salivary gland cells by Ritossa, (1962) and the name was given by Tissieres et al., (1974) according to the molecular mass. Besides the heat shock, several other stimuli can induce and increase synthesis of HSPs, such as amino acid analogs (Kelley and Schlesinger, 1978; Li and Laszlo, 1985), glucose analogs (Pouyssegur et al., 1977), heavy metals (Levinson et al., 1980), protein kinase C (PKC), Ca$^{2+}$ increasing agents (Ding et al., 1996), ischemia, sodium arsenite (Johnson and Somori, 1980), SbIII (Brochu et al., 2004), microbial infections, nitric oxide, hormones, and antibiotics. HSP70 is a molecular chaperone which plays an important role in protein folding and assembly of polypeptides within the cell. When cells are exposed to the stressed condition, the proportion of misfolded proteins (MFPs) suddenly increases and the cell reacts by synthesizing HSPs to assist those proteins in refolding. The stress response is controlled primarily at the transcription level by a heat shock factor (HSF) (Pockley, 2001). The regulation of heat shock protein has been well described in Fig. 10.

9.1 Molecular feature

Heat Shock protein (HSP70) consists of three domains, an N-terminal ATPase domain, a central substrate binding domain and a C-terminal domain (Erbse et al., 2004).

**ATPase domain:** ATPase domain of HSP70 is formed by the union of two domains of similar fold forming a large cleft with an ATP binding site at the bottom (Bork et al., 1992; Flaherty et al., 1990). Both the domains are composed of two subdomains, Subdomains Ia and Iia with an equal number of foldings.

**Substrate binding domain:** The substrate binding domain consists of a β-subdomain and C-terminal α-helical subdomain. Two pairs of inner and outer loops protrude upwards from the β-sandwich and form a cavity, where peptide protein is bound (Zhu et al., 1996). There is a hydrophobic pocket that accommodates a single hydrophobic side chain and an arch formed by residues methionine and alanine that encloses the substrate backbone.
Figure 10: Transcriptional regulation of HSP70 gene by Heat Shock Factor (HSF). Heat Shock Factor (HSF) is present in cytoplasm as a latent monomeric molecule that is unable to bind to DNA. Under stressful condition (e.g. SbIII treatment), large number amount of non native proteins formed which lead to phosphorylation (P) and trimerization of HSF. The trimers translocate to the nucleus, bind the promoter regions of HSP70 and other genes and mediate transcription of HSP70 gene. Diagrams are based on the references (Review by Pockley A.G. 2001), http://www-ermm.cbcu.cam.ac.uk
**C-terminal domain (EEVD motif):** In the folding of proteins such as steroid hormone receptors, HSP70 and HSP90 interact with cofactors such as Hop (HSC70-HSP90 organizing protein), which contain tetra-tricopeptide repeat (TPR) domains (Brinker et al., 2002). TPR motif contains two antiparallel α-helices such that tandem arrays of TPR motifs generate a right-handed helical structure with an amphipathic channel that might accommodate the complementary region of a target protein (Blatch and Lassle, 1999). The TPR domain interact with the C-terminal domain of HSP70 (Brinker et al., 2002). The TPR I domain of HOP recognizes the C-terminal seven amino acids of HSP70 (PTIEEVD). In eukaryotes, EEVD motif is involved in the intramolecular regulation of HSP70 function and intermolecular interaction with HDJ-1 (Freeman et al., 1995).

### 9.2 Therapeutic role of HSP70

All living cells respond to a variety of stresses by the rapid synthesis of heat shock proteins (Kregel, 2002). Over expressed heat shock protein (HSP70) are able to interact with various proteins and peptides shared by major histo-compatibility complex molecules. Because of this ability HSP70 is becoming new immunotherapeutic targets of cancers and infections. During various kinds of insults (e.g. SbIII stress) large amount of unfolded or misfolded proteins are formed. Such proteins have exposed hydrophobic segments that render them prone to aggregation. Protein aggregates are thought to be toxic to the cell (Taylor et al., 2002). Hence, abnormal proteins are either kept soluble by molecular chaperones or quickly degraded by the ubiquitin/proteasome system to avoid aggregation (Hershko and Ciechanover, 1998). Under pathological conditions, the level of abnormal proteins may exceed the ability of the cell to maintain them in a soluble form or degrade them allowing aggregation to proceed (Cohen et al., 1999; Zoghbi and Orr, 2000). Several experiments have shown the beneficial effects of HSP70 overexpression in such conditions such as; induction of HSP72 helps the survival of neurons after stroke (Yenari et al., 1998) as well as improves the efficiency of tissues transplantation (Perdrizet et al., 1993). It helps in neuro-regeneration of diseases like Alzheimer’s, Parkinson’s, Huntington’s or prion disease, as well as trauma; where the accumulation of misfolded proteins is the major cause of neurodegeneration (Carmichael et al., 2000;
Kalmar et al., 2002; Kalmar et al., 2003; Sittler et al., 2001; Warrick et al., 1999). Hsp70 has the potential to protect the brain from ischemic injury (Giffard et al., 2004). The synthesis of HSP70 is associated with protection against light-induced damage to the retina (Barbe et al., 1988) and ischemia reperfusion injury to the heart (Bernelli-Zazzera et al., 1992; Currie, 1988), liver (Bernelli-Zazzera et al., 1992; Buchman et al., 1989; Buchman et al., 1990; Cabin and Buchman, 1990) and kidney (Sakakibara et al., 1992). These evidences suggest that heat shock proteins play an important role to protect cells from damage generated by a variety of stresses.

Heat shock proteins are currently being considered for the potential treatment of diseases involving protein aggregation and misfolding like neuro degenerative diseases (Bonini, 2002). In present context, two types of drugs have been developed targeting overexpression of heat shock proteins. Type I drugs impose a certain level of stress on the cells which provokes induction of HSP70 for e.g. Stannous chloride in tissues transplantations (House et al., 2001), Geranyl-geranyl acetone in cerebral ischemia (Yasuda et al., 2005) and carbenoxolone in ulcer (Nagayama et al., 2001). Type II drugs help to provoke the natural HSP70 induction in cells by natural stimuli. These drugs are called chaperone co-inducers which selectively interact with cells that are in danger. Aspirin (Jurivich et al., 1992) and Bimoclomol are the common example of type II drug. The basic mechanism of these drugs is to induce Hsp70 synthesis by perturbing various membrane structures and helping the release of putative lipid-signaling molecules as well as by prolonging binding of HSF-1 to the heat shock elements on the DNA (Hargitai et al., 2003; Kieran et al., 2004; Torok et al., 2003; Vigh et al., 1997).

In present context, several reports have shown the co-relation between overproduction of Hsp70 and increased drug resistance in cancer. HSP70 overexpression leads to increased cell proliferation and resistant to anticancer drugs (Jaattela, 1999; Nylandsted et al., 2000). Inhibition of HSP70 synthesis as well as induction of apoptosis by treatment with quercetin combined with hyperthermia was reported to be confined to leukemic cells and not to normal hematopoietic progenitor cells. However, HSP70 inhibitors have not been tested in cell or animal cancer models and very few HSP70
inhibitors have been identified. Hence, HSP70 is becoming an emerging therapeutic target to overcome against the drug resistant in cancer and other infectious diseases.

9.3 Cytoprotective role of HSP70

*Leishmania* parasites parasitize the tissue macrophages of their mammalian hosts where they survive and proliferate inside the phagosome. In the course of their digenetic life cycle, sudden increase in temperature becomes a key trigger for the development intracellular amastigote from promastigotes (Zilberstein and Shapira, 1994). Such condition causes stress in the cell and increased synthesis of intracellular HSP in order to provide cellular protection and maintain homeostasis. In some *Leishmania* species elevated temperatures alone can induce stage development (Bates, 1993; Pan et al., 1993). However, in *Leishmania donovani*, a heat shock alone is not sufficient and must be complemented by a shift of pH into the acidic range similar to the conditions inside the phagosomes of mammalian macrophages to induce cellular differentiation towards the amastigotes (Lawrence and Robert-Gero, 1985; Zilberstein and Shapira, 1994). The increased synthesis of heat shock proteins during adverse conditions is correlated well with the acquisition of thermotolerance to various kinds of lethal stresses. Heat shock protein protects organisms not only from lethality but also in cyto-differentiation during the life cycle of the parasites (Lawrence and Robert-Gero, 1985).

HSP70 expression was found to be constitutive even at low temperatures in promastigotes of *L. major* (Smejkal et al., 1988). In *T. brucei*, transcripts of HSP70 and HSP83 were 25- to 100-times more abundant in trypomastigotes at 37°C than in insect stages, 22°C (Van der Ploeg et al., 1985). Furthermore, it has been shown that *HSP70* and *HSP83* genes are induced prior to the morphological changes and remain constitutively expressed in *Leishmania* amastigotes. Thus the presence of HSP prior to transmittance may pre-adapt the parasite for additional stress in the mammalian host (Shapira et al., 1988).

It has been proposed that the heat shock response of parasites plays an essential role during host invasion (Maresca and Carratu, 1992; Polla, 1991; Watson, 1990). At the onset of host invasion, the changes in environment temperature becomes abrupt so
that parasites may not get time to induce a thermotolerant state (Maresca and Kobayashi, 1994). Thus, the entire cellular apparatus such as mRNA maturation machinery, membrane structures, protein folding and transport must remain functional to allow the organism to survive and undergo proper morphogenic adaptation.

10.0 Rationale of the present study

At present, leishmaniasis is re-emerging and spreading worldwide. No effective vaccines against the disease has been developed yet. Hence treatment relies exclusively on chemotherapy. Treatment options for visceral leishmaniasis are limited and there is further limitation of available drugs in terms of either cost or toxicity (side effect).

Pentavalent antimonials are still the drug of choice in the treatment against all forms of *Leishmania* infections throughout the world, except Bihar state of India and some adjoining districts of Nepal. Leishmaniasis treatment with antimonial drugs is threatened in VL endemic regions due to emergence of increased drug resistant parasites. A number of candidate genes associated with resistance to antimonials have been identified on *Leishmania* laboratory mutants in which resistance was induced *in vitro* in the presence of antimonial drugs. Drug resistance mechanism described on the laboratory mutants not necessarily represents the natural drug resistance in the field isolates.

Hence, the present research work is aimed to characterize and validate the identified genes associated with antimony resistance in the clinical isolates.
11.0 Objectives of the present study

To characterize and validate antimony resistance associated genes in clinical isolates in order to develop potential antimony resistance biomarkers for visceral leishmaniasis.

1. To characterize the aquaglyceroporin (*AQP1*) gene in antimony resistance clinical isolates and to explore its role in drug uptake and development of resistant phenotype.

2. To understand the molecular mechanism of natural antimony resistance in clinical isolates of *Leishmania donovani* and identify potential antimony resistance markers.

3. To characterize the role of HSP70 in antimony resistant clinical isolates of *L. donovani*. 