Chapter 1

Introduction and Review of Literature
Parasitic diseases continue to present a major health burden on a global scale. Leishmaniasis is a very widespread disease which contributes significantly to this category of diseases. The parasite *Leishmania* is the causative agent of human leishmaniasis, diseases transmitted by the blood sucking *Phlebotomine* sandfly. Commonly, this genus exists as an extracellular, motile, flagellated, slipper-like promastigote form in the digestive tract of the insect vector and an intracellular, non-motile, amastigote form in the macrophages of the susceptible, vertebrate host (Chang and Bray, 1985).

Leishmaniasis has a global distribution and multiple manifestations depending on the species of the parasite involved in causing the disease. The parasite *Leishmania* causes three major forms of leishmaniasis: cutaneous leishmaniasis or oriental sore, mucocutaneous leishmaniasis or espundia and visceral leishmaniasis or Kala-azar. Of all these three forms of leishmaniasis, visceral leishmaniasis is fatal if allowed to go untreated. All the above three forms together pose a great threat to human health, particularly in developing countries.

In India, this disease accounts for a large number of deaths in regions of Bihar and West Bengal. Pentavalent antimonials have been the chemotherapeutics used till date against Kala-azar (Berman, 1988). However, the widely increasing incidence of this disease throughout the world and particularly in India; and the development of resistance to commonly used drugs has led to the need to identify novel chemotherapeutic targets in the parasite.

The trypanothione pathway is exclusively present in the trypanosomatids (trypanosomes and leishmanias). Owing to this feature, this pathway can be successfully exploited as a chemotherapeutic target in leishmaniasis. This pathway combines the polyamine and the glutathione biochemical pathways (Fairlamb, 1986; Fairlamb and Cerami, 1992) into a compound called trypanothione. The present work deals with the use of metabolic inhibitors targeting some of the important enzymes of the trypanothione pathway in an effort to devise a chemotherapeutic strategy against the parasite. The probable mechanisms of action of these compounds both on the parasite as well on as the host macrophages *in vitro* have also been worked out. Signal transduction pathways
operative in macrophages in response to parasite treatment have also been explored. Another aspect of this study involves elucidating the mechanism of drug resistance in *Leishmania donovani*.

1.1. Geographic distribution and incidence of visceral leishmaniasis

Visceral leishmaniasis or Kala-azar is a major public health problem in different parts of the world and especially in parts of India. As per recent WHO reports, of the estimated four hundred thousand new cases registered annually throughout the world, India is believed to contribute a major share. Bihar and West Bengal have been consistently endemic for this disease; which is gradually but steadily spreading to newer areas and affecting more people. The situation is made more grave by the fact that children are more susceptible to this disease.

Visceral leishmaniasis is not new to India. It has been endemic in the eastern part of the country for more than a century. The first recorded epidemic of Kala-azar was in 1824 in the Jessore District of pre-partition Bengal. There were epidemics at close intervals in Bengal, Bihar and Assam up till around 1955, when the incidence of Kala-azar suddenly declined. The disease seemed to have virtually disappeared in India until it staged a comeback in 1974 in four districts of Bihar. The problem of Kala-azar is presently limited to parts of Bihar and West Bengal. The rise in the incidence is attributed to inadequate vector control and resistance to currently used antimonial compounds (Sehgal and Bhatia, 1988). The present distribution of the disease in India and in the world has been depicted in Figure 1.

In the global scenario, leishmaniasis has been classified by WHO as one of the six major tropical diseases. Estimated figures vary from 400,000 to 12 million cases causing at least 1000 deaths per year. The global distribution pattern of visceral leishmaniasis is depicted in Figure 1. The number of deaths caused by this disease are estimated to be around 40,000 in the last five years and about 25% people are believed to be affected. Eastern Sudan is in the throes of an epidemic that started in 1989 (TDR news, 1993) and Abaroba in Ethiopia represented a major disease focus from 1983-1987, with an annual incidence of approximately 5% of the total population (Ayce and Ali, 1984).
1.2. The causative agent

Leishmaniasis is caused by *Leishmania*, a parasite belonging to the group of trypanosomatid, flagellated protozoans. There are three major forms of leishmaniasis depending on the clinical manifestation of the disease.

1.2.1. *Cutaneous leishmaniasis or Oriental sore* : This form of leishmaniasis is caused by *Leishmania tropica, L. major, L. aethiopica* and several subspecies of *L. mexicana*. It is characterized by cutaneous, dermal lesions on the body of the patient, caused by the survival of the parasite within dermal histocytes of the skin (Fig. 2a).

1.2.2. *Mucocutaneous leishmaniasis* : Espundia or mucocutaneous leishmaniasis is caused by *Leishmania mexicana* and *L. braziliensis*. The parasite affects the mucocutaneous tissue of the nose and mouth parts and leads to gross disfigurements in these areas (Fig. 2b).

1.2.3. *Visceral leishmaniasis or Kala-azar* : *Leishmania donovani* is the causative organism for this form of leishmaniasis. The parasite survives and multiplies in the macrophages of the reticuloendothelial system. This disease, unlike the other forms of leishmaniasis, is fatal if allowed to go untreated. The common symptoms include high fever, loss of appetite, hepatosplenomegaly; and darkening of the skin at the later stages of the disease (Fig. 2c). About 5% visceral leishmaniasis patients develop post kala-azar dermal leishmaniasis (PKDL) in a years time (Napier and Dasgupta, 1931; Singh and Sundar, 1996). PKDL is characterized by the appearance of depigmented patches of skin which progress to diffuse nodular lesions over the body. It is generally non-healing but is not fatal. PKDL cases serve as reservoirs for further infection to other subjects (Fig. 2d).

1.3. Life cycle and Biology of the parasite

*Leishmania donovani* exists in two distinct morphological forms - a motile, slipper-shaped, flagellated promastigote form, which survives in the digestive tract of the *Phlebotomine* sandfly vector and a non-motile, non-flagellated amastigote form which lives in the macrophages of the susceptible,
vertebrate host. A third metacyclic form of the parasite has also been recently observed.

1.3.1. The promastigote form

The promastigote form of the parasite is spindle shaped, measuring 15-25 μ in length and 1-2 μ in breadth. The nucleus is present in the centre of the body and the kinetoplast is present anteriorly. The flagellum, which is 15-25 μ in length, is also present at the anterior end, projecting from a pocket (Chang and Bray, 1985).

1.3.2. The amastigote form

The amastigote is a rounded cell about 2-4 μ in size, containing a round or ovoid nucleus that lies either in the centre or along the side of the cell. The kinetoplast lies at right angles to the nucleus. It comprises a parabasal body and a dot like blepharoplast, with a delicate thread connecting the two. The axoneme arising from the blepharoplast extends to the anterior tip of the cell. It represents the root of the flagellum. A clear vacoule lies along with the kinetoplast (Chang and Bray, 1985).

1.3.3. The metacyclic form

This form of the parasite occurs in the sandfly vector shortly before it is transmitted to the host. Morphologically the parasite is similar to the promastigote form; only it is shorter and more motile than the latter. The abundant form of lipophosphoglycan present at this stage suggests that this is the most virulent form of the parasite.

At the ultrastructural level (Fig.3), most of the organelles present are similar to those present in other trypanosomatids. Three unique features that are present in Leishmania are - the plasma membrane and the flagellar reservoir, the microtubular systems and the mitochondrion-kinetoplast complex (Chang, Fong and Bray, 1985).

The plasma membrane of the Leishmaniae can be separated into three sections. At the anterior end of the parasite, it is invaginated to form a reservoir, in which the flagellum emerges from the reservoir lining membrane above the basal
body. The flagellum extends up to and beyond the opening of the flagellar reservoir for amastigotes and promastigotes respectively. Desmosomes are present at the membrane junction between the flagellum and the opening of the flagellar reservoir. Secretory and endocytic activities are limited to the reservoir lining membrane, which apparently gives rise to or provides the continuity between the plasma membrane of the flagellum and of the cell body.

There are four functional types of microtubules in *Leishmania*. These are subpellicular, flagellar, mitotic and reservoir-associated or cytopharyngeal microtubules. The subpellicular microtubules lie under the cell body section of the plasma membrane and are responsible for providing rigidity and shape to the cytoskeleton of *Leishmania* and other trypanosomatids. The flagellar microtubules are confined to the flagellar region of the parasite and their structure is more or less identical to that in other organisms with flagella as the organelle of cell motility. In line with the observations of other trypanosomatids, mitotic and reservoir-associated microtubules of *Leishmania* probably function in the control of their nuclear division and endocytosis respectively.

The leishmaniae have a mitochondrial-kinetoplast complex. The single mitochondrion in all trypanosomatids branches into many sections, forming a reticulum that often lies below the plasma membrane - subpellicular microtubular system. The ubiquitous kinetoplast is a section of the mitochondrion, located just below the basal body of the flagellum. The mitochondrial DNA in the form of maxicircles and minicircles is condensed into regularly arranged arrays of fine fibrils in the kinetoplast. Amastigotes resemble promastigotes ultrastructurally, except for having less number of certain cell organelles eg. subpellicular microtubules, shorter flagella (concealed within the flagellar reservoir) and no flagellar paraxial rod. Promastigotes of some *Leishmania* species may also have no paraxial rods.

The life cycle of the parasite (Fig.4) begins when the *Phlebotomine* sandfly bites an infected host. Some amastigotes get ingested along with the blood meal of the fly. These amastigotes migrate to the midgut of the sandfly and get transformed into promastigotes; which subsequently divide by binary fission and get anchored to the surface of the gut epithelial cells. At this stage, the
promastigotes are avirulent. These promastigotes subsequently stop dividing, get detached from the epithelial cells, migrate upwards towards the pharynx of the fly and become virulent. This process is called metacyclogenesis (Sacks, 1989). Thus, when the sandfly bites a new host and regurgitates into the wound, the parasite finds its way into the blood stream of the new host. The promastigotes are taken up by the macrophages of the vertebrate host where they bypass the defence mechanisms of the host. They get transformed into amastigotes and survive within the intralysosomal compartment of the macrophages. The parasites multiply by binary fission until the macrophage becomes full and bursts releasing these amastigotes into the bloodstream. These amastigotes further infect more macrophages and the infection spreads further to encompass the entire reticuloendothelial system of the host.

1.4. Host - parasite interaction and pathogenicity

The parasite resides within the phagolysosomal compartment of host macrophages, cells primarily involved in defence against pathogens (Russell, Xu-Songmei and Chakraborty, 1992). The ability of this parasite to reside within these cells poses many intriguing questions namely,

i) How do *Leishmania* evade the oxygen dependent and oxygen independent microbicidal mechanisms of the macrophage?

ii) How do they alter signal transduction pathways within the macrophage?

iii) What biochemical, morphological and phenotypic changes occur for adaptation of the parasite from the insect vector to the intracellular environment?

All these queries are of great interest if we are to devise a suitable chemotherapeutic strategy against leishmaniasis. In order to address this issue, researchers have studied the surface molecules and receptors involved in macrophage - parasite interactions.
1.4.1. The leishmanial surface molecules that mediate the entry of the parasite within the macrophage

1.4.1.1. Lipophosphoglycan

The leishmanial parasites have adapted themselves so that they may survive and proliferate within the hostile environment encountered during their life cycle. They manage to evade lysis and destruction not only in the gut of the sandfly and in the bloodstream of the vertebrate host but also in the phagolysosome of the host macrophages. This protection is conferred to the parasite by certain molecules present on the cell surface (Chang, Chaudhari and Fong, 1990; Turco and Descoteaux, 1992). Lipophosphoglycan (LPG) is one such molecule. This is a lipid containing polysaccharide which is present all over the surface of the parasite and has multifarious functions. It is involved in attachment to host macrophages (Russell and Talamus-Rohana, 1989), resistance to complement mediated damage (Karp, Turco and Sacks, 1991) and survival in the host phagolysosome (Handman et al., 1986; McNeely and Turco, 1990). It has also been implicated in inhibition of macrophage hydrolytic enzymes (Eilam, El-On and Spira, 1985; El-On, Bradley and Freeman, 1980), protein kinase C (McNeely and Turco, 1987; McNeely et al., 1989), oxidative burst (Buchmuller-Rouiller and Maüel, 1987), c-fos gene expression (McNeely and Turco, 1987; Descoteaux and Matlashewski, 1989; Descoteaux et al., 1991) and IL-1 production (Reiner, 1987; Reiner, Ng and McMaster, 1987). LPG causes downregulation of TNF receptors (Turco and Descoteaux, 1992), scavenging of macrophage toxic oxygen metabolites (Chan et al., 1989; Frankenberg et al., 1990) and inhibition of monocyte and neutrophil chemotaxis (Frankenberg et al., 1990).

This glycosyl phosphatidyl inositol phospholipid has four domains:

i) a phosphatidyl inositol lipid anchor with predominantly C 24 : 0 or C 26 : 0 alkyl chains that is probably conserved in all species of Leishmania

ii) a phosphosaccharide core with a Man(α-1,4)GlcN(α-1,6)myo-inositol-1-PO₄ motif that is present in all GPI anchored proteins seen so far. A terminal Gal(α-1,3)Gal region is also seen within the core, which is the epitope against which antibodies are directed during leishmaniasis. The core is connected to the
lipid anchor with an inositol residue within the latter. The core region shows variability in different species of *Leishmania* observed.

iii) a repeating phosphorylated saccharide region which has a backbone structure of \( \text{PO}_4\text{-}6\text{Gal(β-1→4)Man(α-1)} \). This region is variable in different species of *Leishmania* with differences observed in the type of substitutions seen in the mannose or galactose residues.

iv) an oligosaccharide cap that is present at the non-reducing terminal of LPG. It has galactose or mannose residues. The cap structure varies both within various species as well as during different morphological stages of the parasite.

The complete primary structure of promastigote LPG is shown in Figure 5. The tertiary structure of *Leishmania donovani* shows that the oligosaccharide repeats form a helix with mannose and phosphate residues towards the inside and galactose residues towards the outside of the helix. Molecular modelling studies predict that LPG coats the membrane of the parasite surface and forms a diffusion barrier for antibodies directed by the host immune system against the parasite (McConville, 1991; Turco and Descoteaux, 1992).

Lipophosphoglycan of amastigotes (Fig.6) is different from promastigote LPG mainly in the lipid anchor region. The core region and the backbone phosphosaccharide repeats are mostly conserved in the promastigote and amastigote forms. The cap region predominantly shows \( \text{Gal(β1→4)Man(α-1)} \) structure. However, amastigote LPG is expressed at much lower levels as compared to promastigote LPG (McConville and Blackwell, 1991).

1.4.1.2. GP63

This is a 63 KD glycoprotein that occurs in large abundance in all species of *Leishmania* and has been identified as a major surface antigen of promastigotes (Fong and Chang, 1982; Chang, Chaudhari and Fong, 1990). It is a metallo-proteinase with a conserved Zinc-binding region (Etges, Bouvier and Bordier, 1986; Bouvier *et al.*, 1989; Chaudhari *et al.*, 1989). GP63 has been suspected to be one of the ligands involved in macrophage binding (Chang, Chaudhari and Fong, 1990).
1.4.2. The macrophage surface molecules that mediate attachment and internalization of the parasite (Chang, Chaudhari and Fong, 1990)

1.4.2.1. Mannose-fucosyl receptor

This receptor binds specifically to ligands having terminal L-fucose or L-mannose groups. This interaction is a serum independent interaction that follows saturation kinetics, is partially inhibited by these sugars and requires calcium (Lehrman, Haltiwanger and Hill, 1986). LPG and GP63 are the ligands that have been suspected to be involved in these interactions. Wilson has demonstrated the binding of LPG to the mannose receptor of macrophages (Wilson and Pearson, 1986).

1.4.2.2. The C3b receptor

This receptor is supposed to be dependent on the amount of serum present. This macrophage-parasite interaction occurs when the complement factors get adsorbed on the parasite surface and the macrophages bind via their C3b receptors to the parasite (Wilson and Pearson, 1988). The protease activity of GP63 has been implicated in cleaving C3 into C3b and C3a (Etges, Bouvier and Bordier, 1986; Wright and El Amin, 1989) and it may also be involved in direct binding to the macrophage through its mannose moieties.

1.4.2.3. The fibronectin receptor

The fibronectin receptor has also been thought to play a role in leishmanial binding and uptake. However, this receptor is of lesser importance than the C3b or mannose-fucosyl receptors (MFR) since it is not specific to the macrophages and promotes rather than actively mediates phagocytosis (Chang, Chaudhari and Fong, 1990).

1.4.3. Other proteins mediating macrophage-parasite interactions

Another protein has been recently implicated in binding to Leishmania donovani lipophosphoglycan and increasing the uptake of the parasite into human macrophages (Culley et al., 1996). This is the C-reactive protein (CRP), a major acute phase protein that binds to promastigotes at the infectious, metacyclic stage of development, at concentrations found in normal human serum. The
phosphosaccharide repeats of the lipophosphoglycan backbone are probably involved in binding to CRP.

In spite of the fact that certain mechanisms involved in macrophage-parasite interaction have been elucidated, the exact molecular interactions involved are not absolutely clear.

1.5. Different means adopted by the parasite for intracellular survival within the host macrophages

1.5.1. Leishmania donovani modulates host transductional mechanisms for intracellular survival within the macrophage

In order to survive within the host, the parasite has to subvert the immune response of the host. This is brought about by the modulation of the host transductional mechanism in the parasite's favour (Descoteaux and Turco, 1993). This process begins as soon as the parasite binds to the macrophage receptors. The two basic mechanisms of signal transduction are the protein kinase C (PKC) and the protein kinase A (PKA) pathways modulated by phospholipase C and adenylate cyclase respectively (Alberts et al., 1994). Most intracellular parasites exert their effect on host cells via the PKC pathway of signal transduction (Segwin, Keller and Chadee, 1995; Vieira, de Carvalko and de Sonza, 1994; Ward et al., 1994). Leishmania is no exception to this rule (Descoteaux et al., 1991). The parasite alters the PKC pathway and has no effect on the PKA pathway of signal transduction as seen by the use of signal transduction inhibitors and activators (Moore, Labrecque and Matlashewski, 1993; Descoteaux, Matlashewski and Turco, 1992; Descoteaux and Matlashewski, 1989). The portion of the parasite implicated in this effect is lipophosphoglycan (LPG) (Descoteaux, Matlashewski and Turco, 1992).

The parasite leads to inhibition of several macrophage functions by the above means like production of IL-1, expression of the c-fos gene in response to lipopolysaccharide (LPS), the expression of the major histocompatibility complex (MHC) in response to interferon-γ (IFN-γ) and the generation of oxygen radicals following stimulation with LPS (Turco and Descoteaux, 1992). In addition,
interaction or infection of the macrophage with the parasite leads to a homeostatic imbalance of calcium within the host cell (Olivier, 1996). Since calcium is required during signal transduction events, it follows that the sustained elevation of cytosolic calcium that is observed in *Leishmania* infected macrophages (Olivier, Baimbridge and Reiner, 1992; Eilam, El-On and Spira, 1985), is probably responsible for the above effects of LPG. Furthermore, it has been reported recently that the activity of a serine-threonine phosphatase is increased in cells infected with *Leishmania donovani* possibly leading to dephosphorylation of not only second messengers but also various cellular proteins (Olivier, 1996). Under such conditions, the cell is limited to some kind of physiological paralysis, abolishing its ability to defend itself.

Thus it can be seen that the parasite has devised suitable means to defend itself against host microbicidal responses even prior to its uptake by the macrophages. Once the parasite is internalized into the macrophages, other protective mechanisms come into play.

### 1.5.2. The role of heat shock proteins in leishmanial survival

A large number of heat shock proteins are known to be expressed in various *Leishmania* species when they are subjected to a temperature shift under *in vitro* conditions (Hunter, Cook and Hayunga, 1984). Most of these proteins are constitutively expressed by the promastigotes but they become over expressed in heat shocked promastigotes or in amastigotes. Only one of these proteins seems to be immunologically equivalent to Hsp70 protein (Shapira, McEwen and Jaffe, 1988). The exact role of these heat shock proteins in transformation still needs to be elucidated though they seem to reflect another means by which the parasite accustoms itself to the internal milieu of the macrophage.

### 1.5.3. Inhibition of reactive oxygen and nitrogen intermediates in infected macrophages

*Leishmania* infected macrophages show a defective generation of oxygen radicals (Buchmuller-Rouiller and Maüel, 1987). This is a direct consequence of the inhibition of the PKC pathway by the parasite (McNeely *et al.*, 1989). However, this is not particularly significant in light of the fact that nitric oxide and
not oxygen radicals are involved in leishmanial killing (Assreuy et al., 1994; Severn et al., 1993).

Nitric oxide is a physiological messenger molecule as well as a major regulatory molecule in the nervous, immune and cardiovascular systems (Bredt and Snyder, 1994). Nitric oxide is formed by the action of the enzyme nitric oxide synthase. This enzyme converts L-arginine into citrulline and nitric oxide. Nitric oxide synthase exists at three sites in the human body - the brain, the endothelium and the macrophages. The nitric oxide synthase (NOS) activity is constitutive in the brain and the endothelium while it is induced in the macrophages on activation with macrophage activators (Bredt and Snyder, 1994; Leone et al., 1991). An inducible calcium-independent NOS activity has been well characterized in human hepatocytes following treatment with LPS, IFN-γ, TNF-α and interleukin-1β. IFN-γ is released locally and serves to augment inflammatory responses in specific cell populations close to its release. LPS alone stimulates macrophages only to a limited extent. IFN-γ released by infiltrating lymphocytes can prime the macrophages for a maximal response to LPS. The maximal production of NO is restricted to those cells needed to kill foreign organisms, thereby minimizing damage to adjacent tissue (Bredt and Snyder, 1994).

It has been shown that IFN-γ can induce the production of nitric oxide from L-arginine and thereby mediate the killing of leishmanial amastigotes (Iyengar, Stuehr and Marletta, 1987; Green et al., 1990). The components of the parasite that are responsible for subverting the host nitric oxide synthesis are the glycosylinositol-phospholipids (GIPL’s). LPG, in contrast boosts up the nitric oxide production in host macrophages. The parasite therefore evades this host reaction by shedding it’s LPG as soon as it enters into the macrophages (Proudfoot, O'Donnell and Liew, 1995). This makes an effective host immune response even more essential for parasite killing (Evans et al., 1993) and subsequent cure of the disease.

1.5.4. Cytokine profile in Leishmania infected macrophages

Another means by which the parasite survives within the host cells is by causing down regulation in the macrophage's response to stimulating lymphocytes.
There is a disruption of the normal cytokine profile of these cells. Infected cells can effect the Th2 mediated humoral response but they are deficient in the Th1 mediated cell mediated immunity (Miralles et al., 1994; Sundar, Pai and Lahiri, 1996). There is a marked deficiency in the production and release of IFN-γ, TNF-α and IL-2 (Carvalho et al., 1985) while the levels of IL-4 and IL-10 go up (Karp et al., 1993; Zwingenberg et al., 1990), preventing the macrophage to boost a sufficiently strong microbicidal response.

1.6. Chemotherapy of leishmaniasis

1.6.1. Pentavalent antimonials

The first viable treatment for Kala-azar was devised by Dr. U. N. Bhramachari in 1928. He used a soluble form of antimony namely urea stibamine to treat leishmaniasis (Brahmachari, 1928). This was subsequently replaced by less toxic forms of the drug like Sodium stibogluconate (Pentostam) and Meglumine antimoniate (Glucantime). These pentavalent antimonials inhibit glycolytic enzymes and fatty acid oxidation in amastigotes of Leishmania; leading to a dose dependent inhibition in the net formation of ATP and GTP (Berman, 1988). However, the inherent toxicity associated with antimonial compounds and the development of resistance to these agents (Thakur, 1992) has led to an urgent need for the development of new and better chemotherapeutic agents against Kala-azar.

Several other agents have been subsequently tried against this disease. Some of these inhibitors and their mechanism of action is listed below,

1.6.2. Pentamidine

Pentamidine belongs to the category of drugs used in the second line of treatment for Kala-azar since it is used in Kala-azar cases resistant to antimony. It belongs to the class of aromatic diamidines. Pentamidine acts probably via inhibition of kinetoplast DNA function (Olliaro and Bryceson, 1993). Initial studies showed that this drug was highly efficacious in antimony resistant cases with a cure rate of 98.8% without any relapses (Jha, 1983). However, recent studies show that there is a constant decline in the response rate of pentamidine
In addition to this shortcoming, pentamidine has also been found to cause extreme systemic toxicity leading to diabetes mellitus, hypoglycemia (Jha, Singh and Jha, 1991) and cardiac arrhythmias (Sundar, 1996).

1.6.3. Amphotericin B

Amphotericin B is an antifungal agent. The presence of fungus-like sterols in *Leishmania* form the basis for the use of this compound against Kala-azar. This agent inhibits sterol synthesis in leishmanial amastigotes, leading to an increased membrane permeability in the same (Berman, 1988). This drug though successful in antimony refractory cases, has several toxic effects. These include electrolyte imbalance, hepatic and renal toxicity, cardiac arrhythmias and nephrotoxicity (Bryceson, 1987; Sundar, 1996).

Recently lipid-associated amphotericin B has been introduced into the market. The rationale for using this is that the lipid component of the drug will recognize lipid receptors on specific cells like macrophages, sequestering the drug at the intracellular site of infection. This would reduce the toxicity and increase the therapeutic index of the drug. This has indeed been found to be the case in hamsters. However, clinical trials are still on with this formulation and results are awaited to determine the relative efficacy and toxicity of this compound over amphotericin B (Olliaro and Bryceson, 1993).

1.6.4. Aminosidine

Aminosidine or paromomycin is an aminoglycoside. It is used either alone or in combination with antimonials. Pilot studies with the drug have shown that it has no serious side effects, though nephrotoxicity is common (Sundar, 1996). However, one advantage of this drug is that it also has antibacterial and antiamoebic properties.

1.6.5. Allopurinol

Allopurinol is a purine (hypoxanthine) analogue. This analogue hydrolyses to allopurinol riboside, which is an analogue of inosine. These allopurinol ribosides incorporate into the RNA of *Leishmania* instead of adenine and hamper protein synthesis, thus killing the parasite (TDR news, 1991). The use of this compound is
limited due to the fact that it has to be used in combination with antimony and the results obtained are not very consistent.

1.6.6. WR 6026

This compound is a primaquine analogue that has been found to be effective against Kala-azar in some animal models. However, clinical trials in Kenya and Brazil have shown that it is not very effective in vivo in humans (Sundar, 1996).

1.6.7. Fluconazole and other antifungal agents

Fluconazole is an antifungal agent that acts by increasing methylated sterols and decreasing demethylated sterols in leishmanial promastigotes and amastigotes (Berman, Holz and Beach, 1984). Clinical trials with this drug have shown that though it is successful initially in curing visceral leishmaniasis but there is relapse of the disease in all cases, indicating that trials need to be done with either higher doses or to give this drug in combination with other antileishmanial agents in order to achieve a proper chemotherapeutic response (Sundar, 1996). Other antifungal imidazoles that have been tried against the Leishmania are clotrimazole, micorazole, ketoconazole etc. These have also been found to have antileishmanial properties (Berman, 1981; Uraiyo and Zaias, 1982).

Despite the fact that such a large number of antileishmanial agents have been synthesized and used in vitro and in vivo, a need can still be felt for a viable alternative to the currently used antimonial compounds pentostam and glucantime. Thus, efforts are on to develop a rational approach towards leishmanial chemotherapy.

1.7. The polyamine and linked thiol pathway as a specific target for chemotherapy in parasitic protozoa

The development of a rational approach towards chemotherapy involves several steps like identifying features of the parasites' biochemistry or biology that differ from the human host sufficiently to serve as a drug target, isolating and characterizing drug targets at the molecular level and using specific inhibitors that
can serve as lead compounds for further development. The ideal targets are those that are essential for the parasites' survival and absent or non-essential in the host. However, since this is not always possible, the next best targets to use are those, which though common to both systems, are organized into pathways peculiar to each organism or which have diverged sufficiently to warrant their use as chemotherapeutic targets.

A variety of such chemotherapeutic targets have been elucidated in the parasite and the trypanothione pathway is one such target (Fig. 7). Trypanothione is a compound that is present exclusively in the trypanosomatid group of protozoa, including *Trypanosoma, Crithidia* and *Leishmania*. Some very important functions that are essential for the parasites' survival have been ascribed to this compound like maintenance of thiol redox, protein folding, oxidant defences, defence against xenobiotics and heavy metals, coenzyme functions and regulation of polyamine levels (Fairlamb, 1989; Fairlamb and Cerami, 1992).

The trypanothione pathway combines the thiol (namely glutathione) and the polyamine metabolic pathways into a compound called $N^1,N^8$-bis(glutathionyl)spermidine or trypanothione.

**1.7.1. The thiol glutathione (GSH)**

**1.7.1.1. Metabolic functions of glutathione**

Glutathione (L-$\gamma$-glutamyl-L-cysteinyl-glycine; GSH) is a tripeptide thiol present in virtually all cells. It performs a number of vital functions within cells like reduction of disulphide linkages of proteins and other molecules, in the synthesis of deoxyribonucleotide precursors of DNA and in the protection of cells against the effects of free radicals and of reactive oxygen intermediates that are formed during metabolic processes. It also protects cells against heavy metal damage and against xenobiotics. Other functions include maintenance of thiol redox and coenzyme functions. GSH also has a role in inactivation of drugs and metabolic processing of certain endogenous compounds such as estrogens and prostaglandins (Meister, 1983; Meister and Anderson, 1983). Thus inhibition of this thiol represents an important mechanism for metabolic inactivation of cells.
1.7.2.2. Physiological significance of the polyamines

The polyamines have been implicated in a number of vital functions within cells. The roles of polyamines within cells have been elucidated by the study of polyamine mutants and pathway inhibitors. The polyamines are essential for normal growth. Severe polyamine deprivation causes a reduction in the rates of protein and nucleic acid elongation and impairs the fidelity of translation (Marton and Morris, 1987). The precise molecular mechanisms underlying these effects have not been delineated. It has been postulated that spermidine and spermine bind polycationic molecules like DNA, RNA and phospholipids (Igarashi et al., 1982). In vitro these polyamines aid events in DNA replication, transcription and translation (Krasnow and Cozzarelli, 1982). Another role for the polyamines is the synthesis of hypusine, a component of the eukaryotic initiation factor 5A (Park, 1989). Polyamines also play a role in maintaining the integrity of the plasma membrane and have been implicated in intracellular membrane stabilization (Ballas et al., 1983; Chun et al., 1977). They have also been implicated in microtubular functions (Pohjanpelto, Virtanen and Holta, 1981). Earlier reports have shown that depletion of cellular glutathione or cellular polyamines result in disaggregation of microtubules in cancer cells (Liebmann et al., 1993; Pohjanpelto, Virtanen and Holta, 1981). Many observations suggest that polyamines are bound to cell constituents in vivo with most of spermidine and spermine bound to rRNA's and a small amount bound to the DNA (Watanabe et al., 1991). The levels of free polyamines that serve as regulatory effectors are estimated to be quite low and variable depending on the ionic state of the cell (Davis, Morris and Coffino, 1992). This polyamine sequestration could be a means to protect the cell from the toxicity that could result from high intracellular concentrations of the polyamines as also a means to store the polyamines at intracellular sites from where they could be released in large amounts in the event of any emergency.

1.7.2.3. Polyamines in parasitic protozoa

The parasitic protozoans are highly diverse organisms that cause
devastating diseases and contribute heavily to human suffering and economic losses. One of the common features present in these organisms is the polyamine metabolic pathway. Since high levels of polyamines have been correlated with high growth rate and protozoan parasites invade host systems with a very high proliferative activity, it is interesting to explore the relation between their growth patterns and polyamine levels. It is also of interest to study the effect of polyamine inhibitors on the growth of these parasites in vitro and in vivo.

1.7.2.3.1. Polyamines in the malarial parasite

The protozoan parasite *Plasmodium* is responsible for causing malaria in humans, rodents, birds and other animals. Amongst the parasites causing diseases in humans, *Plasmodium falciparum* causes severe and fatal malignant malaria. This is a major problem in a tropical country like India where vector control is inadequate.

A study of the polyamine levels of erythrocytes, the principal cells infected by the parasite, show that polyamines increase dramatically in infected cells as compared to the uninfected RBC's; which have low levels of polyamines (Tadolini *et al.*, 1986; Whaun and Brown, 1985). The polyamine levels in infected RBC's peak at the early trophozoite stage of the parasite and preceed the biosynthesis of proteins and nucleic acids (Assaraf *et al.*, 1984). The principal polyamine of the RBC's is spermidine followed by putrescine and spermine (Assaraf *et al.*, 1984; Rathaur and Walter, 1987; Tadolini *et al.*, 1986; Whaun and Brown, 1985).

1.7.2.3.2. Polyamines in the trypanosomatids

Studies performed on the trypanosomatids like *T. b. brucei*, *T. mega* and *Crithidia fasciculata* by Bacchi *et al.* (1979) showed that the principal polyamine in these organisms was also spermidine followed by putrescine. Spermine was not detected in these cells. It was also shown that the spermidine levels peaked 18 hr before the peak cell population and that the spermidine : putrescine ratio varied between different strains.

1.7.2.3.3. Polyamines in Leishmania

The study of the polyamine levels in *Leishmania* have shown that all the three polyamines are present in the promastigote (Bachrach *et al.*, 1979) and
amastigote (Morrow, Flory and Krasser, 1980) stages of Leishmania donovani, L. tropica and L. mexicana. Both forms have been found to synthesize spermine. Coombs and Sanderson (1985) have found traces of spermine in promastigotes but not in amastigotes of L. mexicana. The spermidine : putrescine ratio varies in all the strains examined. A linear correlation has been observed between polyamine synthesis and specific growth rate of Leishmania promastigotes (Schunur et al., 1979). The ratio of putrescine : spermidine : spermine was around 1.5:1:1 in amastigotes of L. donovani (Morrow, Flory and Krasser, 1980) and around 4:1:0.1 in promastigotes of L. mexicana (Coombs and Sanderson, 1985). These ratios differ from that of African trypanosomes, which have no spermine and have putrescine : spermidine ratios of 1:5 or more (Bacchi et al., 1983). A study of the polyamines in different growth phases of Leishmania have shown that there is an increase in the putrescine levels in the early log phase of the cells followed by rapid accumulation of spermidine (Bachrach et al., 1979; Schunur et al., 1979). In contrast, Coombs and Sanderson (1985) have found that late log phase promastigotes of Leishmania mexicana also have elevated levels of putrescine. Polyamines, especially spermidine and putrescine, accumulate in macrophages of skin and spleen of animals infected with L. tropica major (Bachrach et al., 1981).

1.7.2.4. Polyamine transport in parasitic protozoans

Parasitic protozoa have the ability to transport polyamines into their cytosol from the extracellular medium or the host system. Thus in the event of polyamine depletion within these protozoa, they can overcome the harmful affect of the polyamine inhibitor by transporting polyamines into their systems.

*Leishmania* synthesizes its own polyamines but is also capable of taking up polyamines in the event of polyamine depletion by drugs; as for example DFMO. It takes up spermidine with a seven times higher rate than putrescine; though separate transporters are present for these two polyamines. The putrescine transporter does not recognize spermine but it recognizes spermidine to some extent. The spermidine transporter, on the other hand, recognizes spermine but it does not transport putrescine at all. The functionality of both these transporters is arrested by N-Ethylmaleimide and restored by reduced glutathione (GSH). Drugs
in a complex manner. ODC regulation involves transcriptional (Katz and Kahana, 1987), translational (White et al., 1987) and post-translational events (Welters et al., 1989).

The post-translational regulation of ODC is regulated by another enzyme known as antizyme. Antizyme binds to the ODC and promotes its degradation in a ubiquitin independent and an ATP dependent manner (Murakami et al., 1992; Rosenberg - Hasson et al., 1989). The degradation is carried out by the 26S proteasome.

Mammalian ODC has the property of being a very labile enzyme with a half life of 10-30 min. This ability is conferred to the enzyme by the presence of a PEST (proline, aspartic acid, serine and threonine) motif at the C terminal of the enzyme. The PEST sequence is a common motif found in proteins that have a very high turnover rate (Rogers, Wells and Rechsteiner, 1986). Antizyme also has a PEST sequence at its C terminal end (Xiansiang and Coffino, 1992) reflecting the quick induction patterns and quick degradation of these enzymes; that enable the cell to mount a quick response during stress conditions.

In comparison, trypanosome ODC lacks the PEST sequence and therefore has a relatively longer half-life, making it more susceptible to ODC inhibitors. An analysis of the ODC protein from T. b. brucei has shown an approximate molecular weight of 100,000 and an approximate subunit molecular weight of 45,000. There is 62% homology to the mouse enzyme with the only difference being the addition of an 80 amino acid N-terminal peptide and the absence of a 36 C-terminal peptide in T. b. brucei (Phillips, Coffino and Wang, 1987). The absence of this peptide (The PEST region) causes slower turnover of the T. b. brucei enzyme. The half life of the parasite enzyme is > 6 hr (Bacchi et al., 1989). ODC induction in the parasite (Bacchi et al., 1989) mimics that found in mammalian cells. The induction follows a biphasic pattern with an initial modest increase in activity followed by a decrease and then a major peak of activity within 20 hr. Thereafter the activity rapidly declines probably due to decreased enzyme synthesis and continued rapid cell division. The synthesis of ODC in trypanosomes and mammalian cells requires the synthesis of new mRNA as observed by the use of actinomycin D. ODC induction in mammalian cells is
sensitive to all the three polyamines. In comparison, trypanosome ODC is more sensitive to putrescine as compared to spermidine, showing that the cellular control of this enzyme is not as stringent as the mammalian enzyme.

Analysis of the leishmanial enzyme shows that it has a higher molecular weight than the mammalian and trypanosome enzymes (Hanson et al., 1992a). The N-terminal is approximately 200 amino acids longer than the ODC from other eukaryotes while the C-terminal is truncated as compared to the mammalian enzyme. This C-terminal end contains the PEST sequence (Ghoda et al., 1990; Mukhopadhyay and Madhubala, 1995a). This enzyme shows 40% overall identity to the mouse ODC. However, the leishmanial enzyme, unlike the mammalian enzyme, is insensitive to the presence of the polyamines present in the culture medium. This could be due to the fact that the leishmanial enzyme requires extensive gap introductions for alignment with the mammalian enzyme (Hanson et al., 1992b).

1.8.2. S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase catalyses the second important conversion of the polyamine biosynthetic pathway. It causes the decarboxylation of S-adenosylmethionine to yield an amino propyl moiety, that helps in converting putrescine to spermidine and the latter to spermine under the control of spermidine synthase and spermine synthase respectively. This enzyme has been purified from a variety of sources (Tabor and Tabor, 1984) and though it has been found to vary in its subunit structure, it contains pyruvate as a covalently bound prosthetic group. One pyruvate moiety has been has been found per enzyme subunit.

S-adenosylmethionine decarboxylase is regulated by putrescine, making ornithine decarboxylase the dominant controlling point of the entire polyamine pathway (Kashiwagi, Kobayashi and Igarashi, 1986). Spermidine also activates AdoMetDC; though to a lesser extent than putrescine. Spermine has no direct effect on the enzyme but it inhibits the activation caused by putrescine. The actual mode by which the polyamines regulate AdoMetDC activity is not properly understood. However, there is evidence that regulation of the enzyme could be at the transcriptional and translational levels (Heby, 1981).
In addition to the above means of regulation, S-adenosylmethionine decarboxylase is also regulated at the level of enzyme turnover. The half-life of the mammalian enzyme, like that of ODC, is less than 40 min; allowing it to tightly regulate the levels of the higher polyamines within cells.

The enzyme of *T. brucei* is partially characterized and it has a $K_m$ similar to that of the mammalian enzyme (30 μM). However, it varies from the mammalian enzyme in other respects. Putrescine and Mg$^{2+}$ do not act as stimulators of the enzyme (Bitonti, Dumont and McCann, 1986).

Leishmanial S-adenosylmethionine decarboxylase has not been characterized till date.

### 1.9. Inhibitors of the trypanothione pathway

A variety of inhibitors are available against the trypanothione pathway. Since normal polyamine synthesis is essential for the development of leishmanial parasites, compounds which block this step inhibit the growth of the parasite. Some of the commonly used inhibitors of this pathway have been discussed below.

#### 1.9.1. Inhibitors of glutathione

GSH inhibition can be brought about in a number of ways. One of these is by using substrates of glutathione transferases like α,β-unsaturated carbonyl compounds. One of the most commonly used compounds of this category is diethyl maleate (DEM). DEM has been found to reduce hepatic GSH levels of rats within 30 min of treatment. However, DEM has effects unrelated to GSH depletion and is therefore not used where only GSH depletion is required. Another method to inhibit GSH is to use thiol oxidants that convert GSH to GSSG. The diazenecarboxylic acid derivatives of GSH like methylphenyldiazenecarboxylate is one such compound. This inhibitor does cause GSH depletion but it also leads to undesirable side effects like functional damage and haemolysis of RBC's. In addition these inhibitors cause elevation of GSSG levels in cells leading to activation of the enzyme glutathione reductase, which regenerates GSH at the expense of NADPH. Thus these inhibitors are not generally used (Plummer *et al.*, 2000).
1.9.1.1. L-Buthionine-(S,R)-Sulfoximine (BSO) as an inhibitor of glutathione

Yet another mechanism used to deplete GSH is to block the biosynthesis of this metabolite. GSH biosynthesis is most effectively inhibited by buthionine sulfoximine (BSO), a tightly bound inhibitor of \( \gamma \)-glutamylcysteine synthetase, the enzyme catalysing the first step of GSH biosynthesis (Griffith and Meister, 1979). BSO has been found to have no apparent effects other than depletion of GSH when administered to mice or rats by subcutaneous or intraperitoneal injection (Griffith, 1981).

BSO has been used against certain tumor cells. GSH depletion is effective therapeutically when the normal and tumor cells have markedly different requirements for GSH. Most normal cells have a large excess of GSH but certain tumors and parasites often have levels close to those required for survival (Meister, 1988). Thus depletion of GSH in these cells would probably be more toxic to tumor cells and parasites as compared to normal cells.

The antitrypanosomal effect of BSO was explored by Arrick et al. (1981). They found that when BSO was administered to Female Swiss Webster mice infected with *Trypanosoma brucei brucei* and trypanosomes were isolated from the blood of these mice, they were found to have depleted GSH levels. The parasite was found to be extremely fragile when isolated and examined *in vitro*. The parasite could not be identified in blood samples collected 16-18 hr after BSO administration was begun. In some cases, infection was also cured if plasma BSO concentrations were maintained for 27 hr. In other cases, apparently aperasitemic mice were found to relapse after several days. It was postulated that some parasites persisted after the initial treatment and these multiplied once the BSO was cleared from the plasma. It was also shown that BSO does not react directly with GSH. For BSO to cause GSH depletion the parasites had to lose GSH either through some metabolic process or they had to divide to dilute the GSH pool. Metabolically quiescent trypanosomes were resistant to BSO induced lysis. There was also the possibility of recrudescence of the disease from the central nervous system.
system (Arrick, Griffith and Cerami, 1981).

But despite the fact that the doses of BSO needed for antitrypanosomal treatment are quite high and recrudescence of the disease is a distinct possibility, this inhibitor has no known mammalian toxicity and has little intrinsic chemical reactivity. Also it apparently acts solely via inhibition of GSH biosynthesis and thus does not directly affect other cellular thiols (Arrick, Griffith and Cerami, 1981).

Earlier reports have also described the effect of this inhibitor on nitric oxide levels in macrophages. Macrophages produce high levels of nitric oxide from L-arginine on stimulation by lipopolysaccharide (LPS) or cytokines such as IFN-γ (Stuehr and Marletta, 1985; Stuehr and Marletta, 1987; Hibbs Jr. et al., 1988). The role of nitric oxide in the killing of Leishmania major is well established (Evans et al., 1993; Severn et al., 1993; Stefani, Muller and Louis, 1994). GSH has been shown to have a protective role against the antiproliferative effects of nitric oxide in tumor cells and Leishmania containing macrophages. BSO has been found to reverse the protective effect of GSH by reducing nitric oxide synthase specific activity and nitric oxide synthase protein in cytosolic extracts (Buchmuller-Rouiller et al., 1995; Petit et al., 1996).

Another effect of BSO treatment is that it leads to an increase in oxygen radicals within cells. This has been observed in porcine aortic endothelial cells (Graier et al., 1996). The same group has also observed that simultaneous incubation of the cells with SOD alleviates this effect of BSO.

This inhibitor has not been used previously against Leishmania donovani but our studies show that it is effective against the parasite in vitro. BSO also results in enhanced NO synthesis in murine macrophages.

1.9.2. Inhibitors of polyamine metabolism

1.9.2.1. Inhibitors of ornithine decarboxylase

A large number of inhibitors against the first and the rate limiting enzyme of the polyamine pathway, ornithine decarboxylase are available. Inhibition of ODC leads to a major reduction of putrescine and spermidine but usually to only a small reduction of spermine.
Three major approaches have been used to inhibit ODC: synthesis of analogues of the substrate ornithine and the product putrescine as potential competitive inhibitors, synthesis of molecules capable of interacting with or combining with the cofactor and synthesis of enzyme-activated inhibitors. The former two are reversible inhibitors while the latter belongs to the category of irreversible inhibitors.

The reversible inhibitors of ornithine like α-methylornithine and α-ethylornithine and those of putrescine like 1,4-Diaminobutanone have been used successfully in vitro against the parasite as has been α-hydrazinoornithine; which interacts with the cofactor pyridoxyl phosphate (Bey, Danzin and Jung, 1984; Russell, 1973). However, they are neither potent enough in vivo nor selective enough not to cause any serious side effects (Holutta, Korpela and Hovi, 1981). In addition to this, these inhibitors increase the apparent half-life of ODC (Harik et al., 1974).

In order to overcome the above disadvantages, irreversible inhibitors were designed against ODC. A large number of such inhibitors have been discussed in the literature (Bey, Danzin and Jung, 1984). However, the flourinated derivative of ODC, DL-α- difluoromethylornithine, described by Prakash et al. in 1978, is the most widely used and characterized of these inhibitors. This inhibitor serves as a substrate for ornithine decarboxylase and gets decarboxylated during the reaction. A series of electron rearrangements occur leading to irreversible inactivation of ODC via alkylation of its active centre. Based on its mechanism of action, DFMO is absolutely specific for ODC and does not interfere with any other enzymatic reactions using ornithine as substrate. This inhibitor results in the depletion of putrescine and spermidine in rapidly growing cells, while a slight increase in the concentration of spermine is observed (Pegg, 1986). The cytostatic rather than cytotoxic nature of DFMO can be attributed to the residual pool of spermine within the cells.

DFMO was initially used as an antiproliferative agent in mammalian cell lines (Sunkara et al., 1984). It has subsequently been used against a large number of parasitic protozoans. Bitonti et al. (1987) have shown that DFMO inhibits erythrocytic schizogony of P. falciparum in vitro and reduces spermidine levels in
infected erythrocytes. It has been shown to inhibit the proliferation of *T. brucei in vitro* and has also been used in the treatment of over hundred human patients with *Trypanosoma brucei gambiense* infections (McCann et al., 1981; McCann and Pegg, 1992; Milord et al., 1992). DFMO has been found to be equally effective against *Leishmania donovani* promastigotes *in vitro*. It's effect on these cells is cytostatic rather than cytotoxic in nature (Kaur, Emmett and McCann, 1986). Recent reports have also shown that DFMO is effective in controlling liver and spleen parasitemia levels by 90% and 99% respectively in golden hamsters infected with *L. donovani* when given as a 2% solution for two days after infection (Mukhopadhyay and Madhubala, 1993).

1.9.2.2. Inhibitors of S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase (AdoMetDC) (EC4.1.1.50) is an obligatory enzyme for the biosynthesis of the polyamines spermidine and spermine in higher eukaryotes (Pegg and McCann, 1982) and also in protozoa such as *Trypanosoma brucei brucei* (Bacchi et al., 1979; Dave, Chang and Cheng, 1979).

Inhibition of AdoMetDC leads to a large increase in putrescine levels and decrease in spermidine and spermine levels. The total polyamines actually increase because the increase in putrescine is much more than the decline in the higher polyamines. Thus these inhibitors initially result in less growth inhibition than DFMO.

A large number of S-adenosylmethionine decarboxylase inhibitors are also available though they are not as well characterized as DFMO. These are described below.

Methylglyoxyl bis (guanylhydrazone) is a cytotoxic drug which inhibits cell proliferation (Kay and Pegg, 1973). It is a potent, reversible, competitive inhibitor of AdoMetDC activity (Williams-Ashman and Echerom, 1972). MGBG as well as its numerous analogues possess significant curative properties against most *T. brucei* infections (Bitonti, Dumont and McCann, 1986; Ulrich and Cerami, 1984). It has also been shown to have antileishmanial activity *in vitro*. The reduction in parasitemia is accompanied by inhibition of AdoMetDC activity and spermidine levels (Mukhopadhyay and Madhubala, 1995b). However, MGBG is
not a very specific inhibitor of AdoMetDC. It shares the transport system of the polyamines and may exert its action by this means. In addition, the high toxicity of this compound render it unsuitable as a viable strategy for visceral leishmaniasis.

Berenil is an irreversible inhibitor of AdoMetDC. It has been shown to inhibit AdoMetDC activity from rat liver, yeast and E. coli (Balana-Fouce et al., 1986; Karvonen et al., 1985). Berenil has also been found to exert an antileishmanial effect in vitro (Mukhopadhyay and Madhubala, 1995b). However, the antileishmanial effect of this drug is not solely through the inhibition of polyamine biosynthesis; it has also been shown to block kinetoplast DNA synthesis (Newton and LePage, 1967).

CGP 40215A is a highly specific and potent inhibitor of AdoMetDC that has recently identified by O'Reilly et al. (1993). It has been shown to be a more effective anti-Pneumocystis carinii agent in vivo as compared to MGBG and Berenil (O'Reilly et al., 1993).

CGP 48664A is another specific inhibitor of AdoMetDC. Treatment of L1210 cells with this compound causes a depletion of cellular spermidine and spermine and an elevation of putrescine levels (Svensson, Mett and Persson, 1997).

The CGP compounds have also been found to be highly specific inhibitors of AdoMetDC. Previous studies have shown that CGP 40215A causes a greater inhibition of AdoMetDC as compared to MGBG or Berenil (Kapoor, Mukhopadhyay and Madhubala, 1996) in Leishmania donovani. Earlier studies have shown that both CGP compounds lead to an increase in putrescine levels. CGP 40215A causes an inhibition of spermidine levels while variable observations on the effect of CGP 48664A have been made in the mammalian system with the inhibitor having a modest effect on spermidine to having no effect at all (Manni et al., 1995). The mechanism of action of CGP 48664A may vary in different systems and in some cases may not be completely mediated by the polyamine biosynthetic pathway (Dorhout et al., 1997; Manni et al., 1995; Svensson, Mett and Persson, 1997).
1.9.2.3. Polyamine analogues as inhibitors of the polyamine biosynthetic pathway

Polyamine analogues that have ethyl or benzyl groups on their terminal nitrogen atoms have potent antiproliferative activity and are promising antiparasitic agents (Marton and Pegg, 1995). The bis (benzyl) analogues of spermine are particularly promising in this regard. They are potent antimalarial and antileishmanial agents (Marton and Pegg, 1995). These analogues have been reported to cause the suppression of both antimony-susceptible and antimony-resistant *Leishmania donovani* (Baumann et al., 1990). The bis (benzyl) analogues have also been found to be effective in *in vitro* studies with toxicity increasing with increase in the length of the central methylene chain (Mukhopadhyay and Madhubala, 1995c). These analogues act by inhibiting not only ODC and AdoMetDC activities and polyamine levels but also macromolecular synthesis, indicating that the major mechanism of action may be by disruption of macromolecular biosynthesis and cell death. Thus these analogues probably act by binding to DNA in a manner similar to the natural polyamines (Feurestein, Pattabiraman and Morton, 1986) or by acting as functional intercalating agents such as the diacridine polyamine analogues described by Canellakis et al. (1976). In addition, repression of polyamines by these analogues may be yet another factor involved in slowing the growth of the parasite (Mukhopadhyay and Madhubala, 1995c). There is another advantage in using these inhibitors for leishmanial chemotherapy. Since these analogues are transported by the polyamine transport system, their therapeutic effects are less likely to be blocked by the availability of exogenous polyamines (Marton and Pegg, 1995); which is a major drawback in protozoal chemotherapy. N,N'-bis[3-[(phenylmethyl)amino]propyl]-1,7-diamino heptane (MDL 27695) belongs to the above category of analogues. It has been shown to be a potent antileishmanial agent *in vivo* in golden hamsters infected with *L. donovani*. When MDL 27695 is administered to infected hamsters three times per day for four days with a total dose of 60 mg/kg body weight, liver parasites are reduced by 50% and spleen parasites by 77%. The polyamine content is reduced in the drug
treated animal's liver and spleen, indicating the close correlation between the therapeutic activity of the drug and the polyamine content (Mukhopadhyay and Madhubala, 1993). Baumann et al. (1990) have shown that this compound is equally effective in hamsters against antimony - susceptible or antimony-resistant strains of *L. donovani*.

### 1.9.3. Inhibitors of trypanothione reductase

Trypanothione reductase of the trypanosomatids is analogous to the glutathione reductase of the host system. It is involved in a large number of functions, primary among which is protection against oxidative damage (Fairlamb and Cerami, 1985; Fairlamb, 1988; Fairlamb, 1989). Thus any inhibitor targeted against this enzyme has the ability to inhibit parasite growth (Fairlamb, 1988). Also disruption of the trypanothione gene of *Leishmania* has been found to decrease its ability to survive oxidative stress in macrophages (Dumas et al., 1997).

### 1.10. The models commonly used to observe the effect of inhibitors on parasite growth

A number of models have been used to study the antileishmanial effects of these inhibitors. These are described below.

#### 1.10.1. The in vitro model systems

##### 1.10.1.1. The promastigote model system

The preliminary test to determine the antileishmanial effect, if any, of a compound is to use it against the promastigote form of the parasite in vitro. This is done by adding the drug to a suspension of promastigotes and determining the percentage of live cells after different time intervals of treatment as compared to control values (Mukhopadhyay and Madhubala, 1994).

##### 1.10.1.2. The macrophage-parasite interaction model system

Another model used for testing the antileishmanial effect of a compound is at the macrophage-parasite interaction level in vitro. Macrophages and parasites
are either separately pretreated with the inhibitor and then used in infection assays with their untreated counterparts or the drug is added after the parasite has infected the macrophages and has got transformed into the amastigote form. This assay is helpful in elucidating the probable mechanism of action of the inhibitor at the amastigote stage. It can also determine whether the inhibitor affects the parasite or the macrophage or both and if so, how different is the dosage of the inhibitor required for the two systems (Kierszenbaum et al., 1987).

1.10.2. The in vivo model system

The actual antileishmanial potential of a compound can be determined by using it in in vivo studies in golden hamsters infected with Leishmania donovani since the in vitro effect of a compound may not be mimicked in the in vivo condition. The eight day model of drug treatment is used to determine the antileishmanial efficacy of a compound (Stauber, Franchino and Green, 1958). Golden hamsters are infected with $10^8$ amastigotes on day 0 of the experiment and the drug is administered from days 3 to 7, after which the hamster is sacrificed and the liver and spleen parasitemia is determined and compared to untreated controls.

1.11. Inhibition of microtubular function as a target for chemotherapy in parasitic protozoa

Since microtubules constitute a major part of all cells and they perform crucial functions during cell division, any inhibitor that subverts microtubule function has the capacity of inhibiting cell growth. Microtubule agents belong to two different categories. The first category consists of agents that inhibit microtubular assembly while the second category comprises those agents that bind to microtubules and prevent their disassociation during cell growth. Vinblastine, vincristine and the other Vinca alkaloids belong to the former category while taxol belongs to the latter category of microtubule poisons.

1.11.1. Agents that inhibit microtubule assembly

The Vinca alkaloids like vinblastine and vincristine, colchicine, the benzimidazoles and the dinitroanilines like trifluralin and oryzalin belong to this
category. The former three are anti-mitotic drugs that have been used successfully against cancer while the latter are potent anti-tumor, anti-fungal and anti-parasitic agents (Nare et al., 1994). The Vinca alkaloids inhibit cell proliferation by altering the dynamics of tubulin addition and loss at the ends of mitotic spindle microtubules rather than by depolymerizing the microtubules (Jordan, Thrower and Wilson, 1991). The dinitroanilines have been found to be quite potent antileishmanial agents though their mechanism of action is not properly understood (Chan and Fong, 1990; Chan et al., 1993). Trifluralin that belongs to this category of compounds has been found to be effective against drug resistant Leishmania species (Chan et al., 1995). Oryzalin, another dinitroaniline, has been used successfully as an antileishmanial agent in vitro and it has been found to specifically target Leishmania differentiation (Chan, Triemer and Fong, 1991).

1.11.2. Agents promoting microtubule assembly

Taxol (Paclitaxel) and its analogues like taxotere belong to this category of microtubule poisons. These compounds bind to the microtubules, preventing their disassembly during cell division (Kodali et al., 1994; Makowski, 1995), thereby blocking cells in the G2/M phase of the cell cycle (Schiff and Horwitz, 1980). Mitotic block ultimately leads to apoptotic cell death and appears to be the most potent antitumor mechanism of taxol (Jordan et al., 1996). Taxol has been found to induce the key features of apoptosis like intranucleosomal DNA fragmentation and changes in nuclear morphology in the human neuroblastoma cell line, NB-39-nu (Ogura et al., 1998) and in the MCF-7 cancer cell line (Das, Rao and Madhubala, 1997).

Another means by which taxol has been found to act is by induction of nitric oxide in macrophages. Taxol, like LPS, causes induction of nitric oxide synthase through the PKC pathway of signal transduction in macrophages (Jun et al., 1995). The benzoyl group at the C-3' position of taxol is responsible for this effect of taxol (Kirikae et al., 1996; Kirikae et al., 1998) and the binding of taxol to tubulin is not necessary for macrophage activation. Taxol also causes the induction of TNF-α and the generation of superoxide anions via stabilization of microtubules in murine RAW264.7 macrophages (Pae et al., 1998).
Taxol has been used previously against *T. cruzi* and *Leishmania donovani* promastigotes and it has been found to block replication of the parasite at micromolar concentrations (Moulay et al., 1996; Baum et al., 1981). Drug treated parasites have been found to have multiple cell organelles but they cannot divide fully, confirming that taxol causes inhibition of microtubule disassembly in these cells (Baum et al., 1981).

1.12. Drug resistance

1.12.1. What is drug resistance

Drug resistance implies unresponsiveness to a specific drug. It can be of two kinds - either the host can be unresponsive to the drug or the infective organism can be resistant to the drug. In the first case the detoxification mechanisms of the host cell come into play and in the latter, the parasite adopts various means by which it can neutralize the effect of the drug.

Drug resistance can be classified into two major categories - intrinsic and acquired. The former occurs when the resistance is present in the natural state of the cell as for example the production of antibiotics by the *Streptomyces* species. Intrinsic resistance can be due to either presence or absence of a biochemical feature like the existence of a drug transport protein, the absence of a metabolite pathway, the presence of a drug metabolizing enzyme, the structure of the drug target site, the expression of specific stress response proteins and high repair capacity.

Acquired resistance on the other hand emerges from a population which was previously drug sensitive. This type of drug resistance can arise by a wide variety of mechanisms principal of which is mutation and selection of protective genes. Three major types of genetic change may arise - mutations and amplifications of specific genes directly involved in a protective pathway, mutations in genes which regulate stress-response processes and lead to altered expression of large number of proteins and gene transfer. However, these changes are not mutually exclusive and many or all of them may operate at one time (Hayes and Wolf, 1990).
1.12.2. Mechanisms of drug resistance

Drug resistance is achieved by a large number of mechanisms. The two major mechanisms of drug resistance are biochemical and molecular mechanisms (Hayes and Wolf, 1990).

Biochemical mechanisms by which the cell becomes resistant to a particular drug involves reduced drug delivery, decreased drug uptake, increased drug efflux, reduced metabolic activation of the drug, increased deactivation of the drug, sequestration of the drug to prevent its interaction with the target site, increase in intracellular concentration of target sites, structural alterations in the target site, duplications of the function of the target site and increased repair of the damaged target site.

The molecular basis of resistance to drugs may arise due to gene amplification, gene transfer, gene deletion, point mutations, the loss of cis-acting regulatory elements, the loss or dysfunction of trans-acting factors, transcriptional activation and hypo or hyper methylation.

1.12.3. What is multidrug resistance and how does it arise

Occasionally protection against more than one drug can be seen in the same cell. The two or more drugs may or may not be structurally similar. In the latter case, the cell is termed multidrug resistant.

Multidrug resistance (MDR) is quite a common phenomenon and can be seen in a variety of cells. The most probable reason for MDR is alteration in the drug transport systems of the cell. Indeed, MDR is related to the level of a membrane protein known as P-glycoprotein. This is a 170 kDa protein that probably serves as an energy dependent drug-efflux pump.

P-glycoproteins are encoded by a multigene family with variations in the number of genes within different species. The mammalian P-glycoprotein genes are divided into two major classes - mdr1 and mdr2. The mdr1 family is further subdivided into mdr1a and mdr1b (Kane et al., 1989). The P-glycoproteins encoded by the mdr1a and mdr1b class genes can confer drug resistance but the mdr2 class genes encode a protein that probably does not serve as a drug efflux pump. The physiological function of mdr2 is not yet known.
P-glycoproteins are widely distributed in nature. They are found not only in mammals but are also present in the yeast *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989) and in parasitic protozoa like *Plasmodium falciparum* (Foote *et al.*, 1989) and *Leishmania* tarentolae (Ouelette *et al.*, 1990). The *Leishmania* P-glycoprotein gene (Lt pgpA) is located on the extrachromosomal H-circles of duplex DNA. The amplification of H circles has been associated with methotrexate resistance (White *et al.*, 1988) but Ouelette *et al.* (1990) were unable to demonstrate that Lt pgpA conferred resistance to either methotrexate, adriamycin or vincristine.

The overexpression of P-glycoprotein, however, is not related just to gene amplification. It may also be overexpressed in cells displaying the MDR phenotype without amplification of the gene (Chen *et al.*, 1986). Another gene responsible for multidrug resistance in *Leishmania* is the *ldmdrl* gene, which is homologous to the mammalian *mdrl* gene (Henderson *et al.*, 1992).

MDR is detrimental to cells so agents are required that can reverse the MDR phenotype. These agents are those that can promote drug accumulation and include calcium-channel blockers and calcium-calmodulin antagonists like verapamil, forskolin, nifedipine, trifluoperazine and chlorpromazine. Other agents like the anti-oestrogen tamoxifen and the antibiotic cyclosporin A are also effective in reversing MDR (Hayes and Wolf, 1990; Bradley, Juranka and Ling, 1988).

### 1.12.4. Drug resistance in *Leishmania*

Drug resistance in *Leishmania* has been studied both in natural isolates as well as by raising resistant strains in the laboratory through continuous, selective drug pressure. A large number of cell lines resistant to different drugs have been raised in the laboratory eg. *Leishmania* resistant to methotrexate, to tunicamycin and to DFMO. All these cell lines show amplification of DNA in the form of extrachromosomal circles (Beverley *et al.*, 1984; Garvey, Coderre and Santi, 1985; Hanson *et al.*, 1992b). These amplified DNAs are present as supercoiled or concatenated circles that are highly stable forms. This stability is conferred upon continued selection in the drug and has been postulated to be via acquisition of mitotic-stabilizing sequences in the circular DNA (Garvey and Santi, 1986). Another mechanism of resistance to drugs in *Leishmania donovani* could be via
chromosomal rearrangements.

The gene sequences that get amplified seem to play a role in drug resistance and perhaps mediate other responses that are important for the survival of the parasite. Gene amplification in *Leishmania* has been found to occur in two forms - as extrachromosomal elements and as integrated DNA in a chromosome. The extrachromosomal forms are found to be supercoiled DNA molecules consisting of large inverted repeats that are homologous to chromosomal sequences. The inverted repeat structures may reflect a common mechanism of excision and circularization to form the amplified DNA's in cells (Garvey and Santi, 1986).

DNA amplification, usually as extrachromosomal circular DNA molecules, is a common event found in many *Leishmania* species; and is associated with drug resistance in many cell lines (Beverley, 1991). In most of these systems, drug resistance can be correlated to increase of the copy number of the target gene leading to increase in the mRNA expression and consequent overproduction of the target enzyme. Other mechanisms by which the parasite may become resistant to a drug could be by amplification of genes encoding PGP (P-glycoprotein)-like molecules. P-glycoproteins (PGP's) function as efflux pumps extruding several unrelated, hydrophobic compounds from the cytosol of the cell; thereby preventing their cytotoxic effects (Gottesman and Pastan, 1993). However, multidrug resistance does not necessarily require gene amplification. Increased expression of the multidrug gene without mdr gene amplification is commonly observed in different types of cancers and appears to be a significant marker of clinical drug resistance (Roninson, 1992).

**1.12.5. Drug resistance to microtubular agents**

Drug resistance to microtubular agents has been primarily studied in cancer cells. The mechanism of drug resistance for different microtubular agents is described below,

**1.12.5.1. Resistance to taxol**

Acquisition of resistance to taxol may be mediated through P-glycoprotein or it may be related to some other phenomena as observed in human ovarian
cancer cells (Parekh, Wiesen and Simpkins, 1997).

One mechanism of resistance to microtubular agents is by alteration of the relative tubulin isotype composition. Since, the individual isotypes contribute to differences in microtubule dynamics and drug binding, alteration in the above would change the dynamics and growth patterns of the cells. Resistance to taxol has been correlated with altered expression of M beta 2, the class II beta-tubulin as observed in taxol resistant J774.2 murine cell line (Haber et al., 1995). Resistance has also been correlated with the expression of an electrophoretic variant of alpha-tubulin in Chinese hamster ovary cells (Cabral, Abraham and Gottesman, 1981).

Another mechanism by which a cell can get resistant to taxol; and to adriamycin, is by altering the lipid composition of cells as observed by proton nuclear magnetic resonance spectroscopy in adriamycin and taxol resistant K562 leukemia cells (Le Moyec et al., 1996).

1.12.5.2. Resistance to doxorubicin

Doxorubicin resistance can arise by some other mechanisms like increased expression of P-glycoprotein or multidrug resistance protein, altered glutathione metabolism and altered levels or activity of topoisomerase II (Betekic-Oreskovic et al., 1995).

1.12.5.3. Resistance to Vinca alkaloids

Resistance to vincristine in KB cells has been correlated to increased expression of mdr1 gene and higher production of P-glycoprotein leading to increased drug efflux (Zhang et al., 1994).

Generally resistance to drugs can arise by mutations in the mdr1 gene in cells. However, the specificity of the mutation decides the preferential resistance to the selecting agent. For example, multidrug resistant KB cells selected for colchicine resistance are also resistant to vinblastine and other related drugs, but there is a preference for the former drug in these cells. This preference can be attributed to different mutations in the mdr1 gene of vinblastine selected and colchicine selected KB cells (Choi et al., 1988).
1.13. Rationale of the present study

Visceral leishmaniasis or Kala-azar is one of the most prevalent parasitic protozoan diseases in India today. Of all the forms of leishmaniasis, this disease is usually fatal if left untreated. The toxicity of currently used antimonials and the development of drug resistance has created an urgent need for the development of novel chemotherapeutic agents against Kala-azar.

The naturally occurring polyamines putrescine, spermidine and spermine are ubiquitously distributed in nature. These compounds have been implicated in a wide variety of cellular functions. Thus any disruption in polyamine metabolism would be detrimental to the growth of the cell.

The polyamines are present in both the host and in the leishmanial parasite, but in the latter, they combine with the glutathione pathway to form a novel compound called trypanothione. This compound is present exclusively in the trypanosomatid group of parasitic protozoa, of which Leishmania is a member.

This study deals with the use of inhibitors of polyamine and glutathione metabolism both at the in vitro as well as at the in vivo level in an attempt to devise a suitable chemotherapeutic strategy against Kala-azar. The differential mode of action, if any, of these inhibitors against the host and the parasite has also been studied by using them against a macrophage-parasite interaction model in vitro. The mechanism of action of these drugs on the parasite has also been elucidated. Signal transduction mechanisms induced in host macrophages in response to leishmanial infection have also been worked out.

Since tubulin is one of the most abundant proteins in leishmanial promastigotes, a tubulin inhibitor, taxol was also used as a chemotherapeutic agent against the parasite.

Drug resistance is a common problem in the treatment of leishmaniasis. Thus, it is interesting to know the means by which the parasite develops resistance to drugs. This thesis also involves the raising of leishmanial strains resistant to taxol in an attempt to study the mechanism by which the parasite acquires resistance to this drug.
Fig.1 Global distribution of visceral leishmaniasis.
Fig. 2 – Pathology of Leishmaniasis (Adapted from Pathology of Tropical and Extraordinary Diseases, an Atlas, Vol. I)

a. A Venezuelan with diffuse anergic cutaneous leishmaniasis.

b. A Brazilian with mucocutaneous leishmaniasis showing deformed nose and lips.

c. Autopsy of a man with visceral leishmaniasis. Note enlarged liver and spleen.

d. A man showing Kala-azar dermal leishmaniasis (PKDL).
Fig. 3 Diagrammatic presentation of leishmanial subcellular structures of [A] Promastigote [B] Amastigote (Adapted from Chang & Bray/Leishmaniasis, 1985).

AF: Paraxial or para-flagellar red; AX: Axoneme; D: Desmosome; R: Flagellar reservoir; VE: Vesicle; BP: Basal plate; BB: Basal body; RA: Reservoir associated (cytoplasmic) microtubules; K: Kinetoplast; G: Golgi apparatus; A: Acanthosome; M: Mitochondrion; V: Vacuole; DV: Dense inclusion vesicle; SR: Smooth endoplasmic reticulum; RER: Rough endoplasmic reticulum; N: Nucleus; NL: Nucleolus; NE: Nucleus envelope; L: Lysosome; SPT: Subpellicular microtubules; MBV: Multivesicular body.
Fig. 4 Leishmanial life cycle in sandfly and in mammalian host. (Adapted from Chang and Bray, 1985)

1. Delivery of promastigotes (proboscis form) into human skin by the bite of sandfly vector; 2. Attachment and engulfment by phagocytosis of promastigotes by a macrophage; 3. Fusion of phagosome containing a promastigote with lysosome in a macrophage; 4. Differentiation of promastigote into amastigote in the phagolysosome of the infected macrophage; 5. Multiplication of an amastigote in a parasite-containing or parasitophorous vacuole; 6. Formation of large parasitophorous vacuole and continuing replication of intravacuolar amastigotes; 7. Rupture of heavily parasitized macrophage and release of amastigotes; 8. Phagocytosis of released amastigotes by a macrophage; 9. Ingestion of parasitized macrophage by sandfly after a blood meal taken from infected person or reservoir animal; 10. Rupture of the ingested macrophage and release of amastigotes in the gut of sandfly; 11. Replication of amastigotes and their differentiation into promastigotes; 12. Replication of promastigotes (termed neptomonads for *Leishmania mexicana* group) in the abdominal midgut and insertion of their flagella into microvilli of the gut epithelial cells; 13. Replication of *L. braziliensis* group in the pylorus and ileum of the sandfly hind gut as promastigotes with broadened flagella attached to the chitinous gut wall via hemi-desmosome; 14. Forward movement of promastigotes to thoracic midgut as heptomonads with broad flagella attached to the chitinous gut wall; 15. Sessile promastigotes with broad flagella attached to the chitinous wall of stomadeal valve; pharynx and buccal cavity (cibarium); 16. Actively motile promastigotes found in the proboscis or mouth part of sandfly.
Fig. 5 Structures of promastigote LPG from three promastigote species of *Leishmania* grown in log to late log phase of growth.
Fig. 6. The complete primary structure of *L. major* amastigote LPG. A PI-lipid moiety anchors a glucosylated phosphorylated saccharide core, which is linked to a series of phosphorylated oligosaccharides that are capped by a neutral disaccharide.
**Fig. 7** Biosynthetic Pathway of Trypanothione.

PUT-Putrescine; SPD-Spermidine; SPM-Spermine; GSH-Glutathione; GSH-SPD-Glutathionyl spermidine; T[S]$_2$-Dihydrotrypanothione; T[S]$_2$-Trypanothione disulfide; GSSG-Glutathione disulfide.

Fig. 8 The molecular structure of three major polyamines in mammalian cells. All of the primary and secondary amine groups are shown in the protonated form, as occur under physiological conditions.
Fig. 9 – The biosynthetic pathway of the polyamines.