Chapter 1

Review of Literature: Leishmaniasis
Leishmaniasis

Parasitic diseases were always a heavy burden for humanity. Protozoan parasites are responsible for several important diseases that threaten the lives of nearly one quarter of the human population worldwide. Leishmaniasis is a disease complex, caused by haemoflagellate obligate intracellular protozoa belonging to the genus *Leishmania*, family Trypanosomatidae of the order Kinetoplastida. *Leishmania* parasites were previously observed by David D. Cuningham in 1885 and Peter Borovsky in 1898. These parasites were mistaken for other protozoa, but later named as *Helcosoma tropica* by James Wright in 1903. Luhe in 1906 renamed it as *Leishmania tropica*. Similarly, the causative agent of visceral leishmaniasis was first named as *Pyroplasma donovani* which was rechristened as *Leishmania donovani* by Ross in 1903 after its discoverers Leishman (1900) from London and Donovan (1903) from Madras who reported the organism independently (Herwaldt, 1999). There are more than 20 different protozoan species of the genus, *Leishmania* that are pathogenic for humans and transmitted by an insect vector, the phlebotomine sand fly.

The leishmaniasis has a long history of its origin. Designs on pre-Colombian pottery and the existence of thousand year old skulls prove that the disease has been present in the Americans for a long time (Manson-Bahr, 1996). It has also been present in Africa and India since the mid eighteenth century (Berhe et al., 1999). It ranks second only to malaria, causing considerable morbidity and mortality in tropical and subtropical regions of the world. The control of leishmaniasis remains a serious problem and with ever increasing cases world wide, it has become a major focus of concern and a serious third World problem afflicting the poorer sections of the society (WHO, 2002).

1.1. Taxonomy

The classification of *Leishmania* was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties and clinical manifestations
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(Marsden and Lumsden, 1971; Bray, 1974; Pratt and David, 1981; Ryan et al., 1990). However, biochemical and molecular analysis showed that pathological and geographical criteria were often inadequate and thus, other criteria such as the patterns of polymorphism exhibited by kinetoplastic DNA (k-DNA) markers, proteins or antigens came to be used to classify *Leishmania* (Arnot and Barker, 1981; Miles et al., 1981; de Ibarra et al., 1982; Handman and Curtis, 1982; Wirth and Pratt, 1982; Brainard et al., 1986; Le Blancq et al., 1986; Travi et al., 2002). A modern scheme of classification of *Leishmania* is shown in Fig. 1.1. All members of the genus *Leishmania* Ross, 1903 are parasites of mammals. The two subgenera, *Leishmania* and *Viannia*, are separated on the basis of their location in the vector’s intestine (Ryan et al., 1990). Rioux et al. (1990) used isoenzyme analysis to define species complexes within the subgenera. Initially, species classification was based on various extrinsic criteria such as clinical, geographical and biological characteristics—for example, *L. guyanensis* (isolated in Guyana), *L. peruviana* (isolated in Peru), *L. infantum* (isolated from a child in Tunisia) and *L. gerbilli* (isolated from gerbils). Since the 1970s, intrinsic criteria such as immunological, biochemical and genetic data have been used to define species of *Leishmania*. Use of these molecular techniques led to the publication of a taxonomic scheme by the World Health Organization (WHO, 1990). New methods of detection, isolation and genetic identification resulted in a massive increase in the number of species described. Today, 30 species are known and approximately 20 are

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**Fig. 1.1: Taxonomy of Leishmania (Based on the scheme published by the World Health Organization [WHO, 1990]).**

(Figures and diagrams are not displayed in text format.)
pathogenic for humans. These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. The validity of the classification scheme, considered by some workers as too arbitrary, has been questioned several times. Debate has centered on *L. panamensis*, *L. peruviana*, *L. chagasi*, *L. infantum*, *L. archibald*, *L. garnhami*, *L. pijanoi*, *L. venezuelensis* and *L. forattinii* (Mauricio et al., 2000; Cupolillo et al., 2001; Sharma et al., 2005). Different studies have already clarified the status of some of these species; for example, *L. chagasi* is accepted as a synonym of *L. infantum* (Mauricio et al., 2000) and *L. peruviana* has been validated as an independent species (Banuls et al., 2000). The other species listed above are still under discussion.

1.2. Geographical distribution

Leishmaniasis has been reported in 88 countries in five continents—Africa, Asia, Europe, North America and South America (22 in the New World and 66 in the Old World) (Desjeux, 2001), 16 are developed countries, 72 are developing, and 13 of them are among the least developed (WHO, 2005). Approximately, 350 million individuals are at risk of this disease and 20 million people are infected worldwide, and an estimated 2.0 million new cases occur each year (Leishmaniasis control, www.who.int/health-topics/leishmaniasis.htm, update 2007) with an incidence of 1.5 million cases per annum of the disfiguring cutaneous leishmaniasis (CL) and 0.5 million cases per annum of the potentially fatal visceral leishmaniasis (VL) (Ashford et al., 1992). However, with increasing travel to and from endemic regions more and more patients with leishmaniasis are seen by physicians in western countries (Herwaldt, 1999; Murray et al., 2000; Guerin et al., 2002). The relevance of this parasitic disease is further stressed out by the rise of *Leishmania/HIV* co-infection in many parts of the world including European countries such as Spain, Italy, France and Portugal where up to 9% of the AIDS patients suffer from visceral leishmaniasis (Berhe et al., 1999).

Over 90% of the global total of VL cases occurs in five countries; India, Bangladesh, Nepal, Sudan and Brazil (Herwaldt, 1999) and 90% of CL cases occur in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. During the past decade there have been epidemics of VL in Sudan (Ashford et al., 1992; Jacquet et al., 2006), northeast Brazil (Costa et al., 1990), Bangladesh and the states of Patna and Bengal in India (Bolognesi et al., 1999). Leishmaniasis is now an emerging zoonosis in the United States (Enserink, 2000; McHugh et al., 2003; Rosypal et al., 2003) and US soldiers and
peace keeping corps currently in the Middle East are experiencing a large outbreak of leishmaniasis with more than 500 parasitological confirmed cases (CDC, 2004).

1.2.1. Leishmaniasis in the Indian subcontinent

1.2.1.1. Historical background

The rich heritage ancient medical literature of India makes no mention of kala-azar, the popular name of visceral leishmaniasis or any disease entity akin to it. Kala-azar is term coined in India and literally mean ‘black fever’ but could equally mean ‘fatal fever’. The earliest available reference on the subject is contained in the annual report of the Inspector General of civil hospitals for 1872, in which he has quoted the civil surgeon of Burdwan district about the disease. The source from which kala-azar was introduced in Indian subcontinent remains a matter of conjecture. The advent of the disease in Bengal coincides with periodic incursions and raids by the Portuguese to the area, and it is possible that they introduced the disease either from their own country or from colonies from Africa. Alternative source could have been linkage with China through British maritime traffic. However, the disease in India is not identical with the disease in any of these areas, the nearest resemblance being with the East African form. Once introduced, the condition being appropriate, the disease possible adapted itself and then established a firm foothold.

1.2.1.2. Current situation in India

In the Indian subcontinent (Bangladesh, Nepal and India), the most common endemic form of the disease is visceral leishmaniasis (VL) or kala-azar or Dum-Dum fever. Kala-azar is present in India for more than 100 years. The first appearance of kala-azar in India was recorded in 1862, when about 75,000 cases were reported from Mohammadapur in Jessore district of East Bengal (now in Bangladesh) (Sen Gupta, 1944; Peters and Prasad, 1983). All the districts north of the river Ganges were affected with Kala-azar. Now the disease has spread southwards up to Darjeeling, Malda, West Dinajpur and Burdwan districts of West Bengal bordering Bihar state. A sample survey in Bihar carried out in 1977, on the epidemic of kala-azar showed an estimated number of 100,000 cases in the state with 4500 deaths (Sanyal et al., 1979); in 1989, 30,000 cases and 450 deaths. In 1990 infected cases reached to 54,000 with 590 deaths and by 1991 the number of cases increased to 250,000 with 75,000 deaths (Thakur et al., 1993). It is obvious that the number of reported cases largely underestimated. Some local surveys revealed that the real prevalence of disease was five times more than what was reported. The situation is particularly grave in the state of Bihar, India, known as the “heartland of kala-azar”. It has recently been posed a
serious threat involving 38 out of 42 districts of Bihar state, 8 districts of West Bengal and 2 districts of eastern Uttar Pradesh (Guerin et al., 2002). At present the disease is present in almost all districts of Bihar, four districts of Jharkhand, five districts of Uttar Pradesh and 10 districts of West Bengal, 40 out of total 54 districts in Bihar are badly affected with VL. The known endemic districts of kala-azar are located north of the river Ganges namely Muzaffarpur, Vaishali, Darbhanga, Samastipur, Madhubani, East Champaran, Sitamarhi, Begusarai, Saran, Saharsa and Purnea (Fig.1.2).

Fig.1.2: Map of resistance levels in the Bihar focus of visceral leishmaniasis, India. Orange-high-level resistance, yellow-mixed resistance, green-no resistance (Trop Med Int Health 2002; 7: 293).

Sporadic cases have also been reported from Gujarat (Gajwani et al., 1967), Kashmir (Jacob and Kalra, 1951) Himachal Pradesh (Gupta and Bhatia, 1975). In Uttar Pradesh occurrence of sporadic cases of kala-azar started in the year 1987 with most of the cases reported so far from this state are imported cases (Thakur et al., 1999) and in West Bengal 9 districts are affected including Malda, Dinajpur and Darjeeling districts. Kala-azar has spread from Bihar to West Bengal, eastern Bangladesh, and eastern Uttar Pradesh and in the northern Nepal. In 2005 the health ministers of three Member States of WHO South-East Asia Region, India, Nepal and Bangladesh, had signed a Memorandum of Understanding pledging to collaborate to eliminate VL from their countries.
Geographical distribution of kala-azar closely coincides with the distribution of insect vector, *Phlebotomus argentipes* and ecological factors (Napier and Smith, 1926) (Shivaramakrishnamaiyah and Ramanathan, 1967) such as:

a) an altitude less than 2000 feet

b) abundant rainfall more than 80 cm. annually and mean humidity of about 70% to 80%

c) alluvial soil

d) temperature below 38°C and above 4°C with diurnal variation less than 10°C

e) abundant vegetation with subsoil water

f) rural setting.

All these conditions prevail in Assam valley, West Bengal, Tamil Nadu and Bihar (Iyer, 1985).

1.3. Disease and its epidemiology

Leishmaniasis is not a single disease but a variety of syndromes that differ remarkably with one another. The WHO considers leishmaniasis as one of the most important parasitic diseases (WHO, 1990).

1.3.1. Various forms of Leishmaniasis

Leishmaniasis is a group of diseases with wide epidemiological and clinical diversity. The leishmaniasis is caused by over 20 species, pathogenic for humans, belonging to the genus *Leishmania*, a protozoa transmitted by the bite of a haematophagous insect vector, the phlebotomine sand fly. Governed by parasite and host factors and immunoinflammatory responses, the clinical spectrum of leishmaniasis encompasses subclinical (unapparent), localised (skin lesions), and disseminated infection (cutaneous, mucosal, or visceral). These wide-ranging differences of clinical manifestations define *Leishmania* virulence (degree of pathogenicity) in human infection. According to the form of the disease, site of infection and species involved, the leishmaniasis can be divided into following general clinical patterns.

1.3.1.1. Cutaneous Leishmaniasis (CL)

CL is commonly known as oriental sore. Its causative agents are *Leishmania major*, *L. tropica*, *L. aethiopica*, *L. infantum* in old World and *L. mexicana*, *L. venezuelensis*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. peruviana* and *L. chagasi* are in New world. It produces skin lesions mainly on the face, arms and legs. It is frequently
self-healing in the Old World but when the lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma. Its most severe form, recidivans leishmaniasis, is very difficult to treat, long lasting, destructive and disfiguring. After recovery or successful treatment, cutaneous leishmaniasis induces immunity to re-infection by the species of *Leishmania* that cause the disease. Most commonly, it is prevalent in Mediterranean Basin, Syria, Arabia, and Mesopotamia, Persia to Central Asia, Central Africa and some parts of Western India.

1.3.1.2. Diffuse cutaneous Leishmaniasis (DCL)

It is difficult to treat DCL due to disseminated lesions that resemble leprosy and do not heal spontaneously. This form is especially related to a defective immune system and it is often characterized by relapses after treatment.

1.3.1.3. Mucocutaneous Leishmaniasis (MCL)

It is also called 'espundia' in South America. Causative Agents of MCL in Old World are *Leishmania aethiopica* (rare), *L. major* and in New World are *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. guyanensis* and *L. panamensis*. The parasite invades the mucocutaneous region of the body and spread to the oronasal/pharyngeal mucosa. The soft tissues and cartilage of the oronasal/pharyngeal cavity undergo progressive erosion. In contrast to cutaneous leishmaniasis, these lesions do not heal spontaneously. Suffering and mutilation are severe and death occurs as a result of bronchopneumonia or malnutrition. There is always a large danger of bacteria infecting the already open sores. Reconstructive surgery of deformities is an important part of therapy.

1.3.1.4. Visceral Leishmaniasis (VL)

Visceral leishmaniasis (VL, kala-azar) is prevalent in 62 countries with an estimated annual incidence of 500,000. In India, the State of Bihar and adjoining areas of West Bengal, Jharkhand and Uttar Pradesh account for about half the world’s burden of VL (Sundar *et al.*, 2006). It is also known as 'Kala-azar' (in India). It is caused by *Leishmania donovani* complex i.e. *L. donovani donovani* (India, Africa), *L. d. infantum* (Middle East and some parts of Asia) and *L. d. chagasi* (South America). These species are morphologically indistinguishable but have been identified by molecular methods, predominantly multilocus enzyme electrophoresis. The disease can present an acute, subacute or chronic evolution, but most infected individuals remain completely asymptomatic (Bittencourt *et al.*, 1995). The asymptomatic individual is characterized by positive serology to *Leishmania* and, possibly, a positive intradermal test. Infected
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individuals can evolve to a subclinical form of VL or directly to an overt form of disease (classical VL). Initially, the disease is characterized by high fever, headache, chill, malaise, dizziness, anorexia, and vomiting and weight loss. In chronic stage the disease is followed by hepatomegaly, splenomegaly, lymphadenopathy, occasional acute abdominal pain, emaciation, anemia, leucopenia, and blackness of skin, hence the name given kala-azar or Black fever. As the disease advances, splenomegaly can increase, causing abdominal distension and pain, which is sometimes increased by concomitant hepatomegaly followed by severe anemia and cachexia. Symptoms and signs of bacterial co-infections such as pneumonia, diarrhea or tuberculosis can confuse the clinical picture at the time of initial diagnosis (Chappuis et al., 2007). It is the most severe form of leishmaniasis and is usually fatal (100% deaths) if left unattended. The incubation period can be months or years and, unlike the cutaneous forms of leishmaniasis, in this disease, the parasite uses the bloodstream to travel and it involves the internal organs such as liver, spleen, lymph nodes, and bone-marrow. After treatment and recovery, the patients may develop chronic cutaneous leishmaniasis that requires long and expensive treatment.

1.3.1.5. Post Kala-azar Dermal Leishmaniasis (PKDL)

Post kala-azar dermal leishmaniasis is a sequel to the infection with *L. donovani*. Its causative agents in Old World are *L. infantum*, *L. donovani*, and *L. tropica* (rare; also may produce the atypical viscerotropic disease) and in New World *L. chagasi* is responsible for this. It is a type of non-ulcerative cutaneous lesion, developed in about 5 to 15 percent of kala-azar patients generally one or two years after completion of antimonial treatment (Rees and Kagar, 1987; Salotra et al., 2006). PKDL in India resembles lepromatous leprosy with verrucous papilomatous, xanthomatous and gigantic nodular forms; while in East Africa it resembles more to sarcoidosis and tuberculosis with popular rash over face or well defined rounded papules (Rashid et al., 1986).

1.3.2. Epidemiology of Leishmaniasis

It has been reported, there are an estimated 500,000 new cases for VL and more than 50,000 deaths from the disease each year (Desjeux, 2004), a death toll that is surpassed among the parasitic diseases only by malaria (WHO, 2002). Both figures are approximations as VL is frequently not recognized or not reported (Collin et al., 2006; Singh et al., 2006). The majority (>90%) of cases occur in just in six countries- Bangladesh, India, Nepal, Sudan, Ethiopia, and Brazil. Severe VL epidemics have been reported in the
past in southern Sudan, in context of civil war and famine, VL killed and estimated 100,000 people out of a population of 280,000 between 1984-1998 (Jacquet et al., 2006). As India, Nepal and Bangladesh harbour an estimated 67% of the global VL disease burden (Hotez et al., 2004), the commitment of the government of these countries to launch regional VL elimination programme is welcome. The target of this programme is to eliminate VL as a public health problem by 2015, by using a local approach to reduce the annual incidence of VL to less than one case per ten thousand individuals. Leishmanias cause considerable morbidity and mortality. Leishmaniasis is a typical example of an anthropozoonosis. The majority of infections are originally zoonotic, although some cases are known of transmission of L. donovani from human to human. The different epidemiological cycles are: (i) a primitive or sylvatic cycle (human infection is accidental, transmission occurring in wild foci), e.g. L. braziliensis; (ii) a secondary or peridomestic cycle (the reservoir is a peridomestic or domestic animal, the parasite being transmitted to humans by anthropophilic sand flies), e.g. L. infantum; and (iii) a tertiary, strictly anthroponotic cycle (in which the animal reservoir has disappeared, or not yet been identified, and the sand fly vectors are totally anthroponotic) e.g. L. donovani. Nevertheless, many unknown factors remain. For example, the main animal reservoir of L. braziliensis is still unknown (Cupolillo et al., 2003). L. tropica was considered to be a strict anthroponosis, but several cases of canine infection have been described (Dereure et al., 1991a; Dereure et al., 1991b; Yahia et al., 2004).

The epidemiology of leishmaniasis in a given area is directly dependent on the behavior of the human and/or animal population in relation to the cycle of transmission. There are a variety of factors that influence the transmission of the disease (Lane, 1993; Kettle, 1995). They are as follows:

- Proximity of residence to sand fly breeding and resting sites
- Type of housing
- Occupation
- Extent of exposure to sand fly bites
- Natural resistance, genetic or acquired
- Virulence of the parasite species
- Zoonotic or anthroponotic reservoirs. It seems that zoonotic reservoirs are particularly stable when wild uncontrolled populations (e.g. rodents) are involved. Up till now it has been observed that humans are not a reliable agent because of death and treatments except
of the chronic condition of PKDL. Nevertheless recent reports about asymptomatic infections in healthy blood donors in France (Le Fichoux et al., 1999) are adding a new parameter to the later

- The vectorial capacity, which is defined as the number of infective bites delivered per human per annum (Dye, 1992)
- Density, seasonality, longevity and flight range of sandfly populations
- Anthropophilia or zoophilia of sandflies and degree of it.

1.4. Entomology

The insect vectors of *Leishmania* parasites are sandflies belonging to the family Psychodidae, sub-family Phlebotominae and genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) with hundreds of species spread all over the world (Volf et al., 1994). Of the 500 known phlebotomine species, only 30 of them have been positively identified as vectors of the disease. Only the female sandfly transmits the protozoa, infecting itself with the *Leishmania* parasites contained in the blood, during the blood meal from human or mammalian host in order to obtain the protein necessary to develop its eggs.

In the Old World (Europe, Asia, and Africa) sandfly vectors belong to the genus *Phlebotomus* and in the New World (America), to the genera *Lutzomyia* and *Psychodopygus*. The gut of the haematophagous insect is a potentially nutrient rich but highly specialized environmental niche, and the successful development of ingested potential pathogens or parasites such as *Leishmania* depends on their ability to avoid or adapt to the dramatic changes in the physicochemical environment accompanying blood-meal and sugar-meal digestion. The strategy of the mosquito-borne malaria parasite is to exit rapidly through the gut epithelium and continue development in the hemocoel. In contrast, African trypanosomes in tsetse flies (Aksoy et al., 2004) and *Leishmania* (Sacks and Kamhawi, 2001) have adapted to remaining and developing in the insect gut. The *Leishmania* parasite is supremely adapted to the gut environment of the sand fly, secreting a unique gel like material composed mainly of a high-molecular-weight filamentous proteophosphoglycan (f PPG). *Leishmania* fPPG serves a dual function, first blocking the fly gut and improving chances for transmission and subsequently aiding survival of the parasite in the mammalian host (Rogers et al., 2002; Warner et al., 2004).

There are different vectors in different regions for a single spp, for example, vectors of *Leishmania donovani* are *Phlebotomus argentipes* in India, *P. chinensis* in China, *P. perniciosus* in North Africa, Italy, France and Portugal, *P. perfiliewi* in Greece, *P. orientalis*
in Sudan, and Ethiopia, *P. martini* in Kenya (Le Blancq and Peters, 1986), and vectors *L. infantum* are *P. erniciosus, P. ariasi, P. perfiliewi* and *P. neglectus*. Sometimes a single species is transmitted by a single vector, e.g. *L. chagasi* is transmitted by sandflies belonging to the genus *Lutzomyia* (*L. longipalps*) (WHO, 1990).

Transmission of parasite may be anthroponotic (from one human to another) or zoonotic (from animal to human). In India, the disease is completely anthroponotic where as in certain parts of the world, there are one or more reservoirs (zoonotic host) e.g. dogs in the Mediterranean region and rodents in South Africa.

### 1.4.1. Distribution of vector in India

*Phlebotomus argentipes* prefers to hot and humid climates in all the VL abundant endemic areas of Bihar, West Bengal, Assam and Eastern Utter-Pradesh. High densities have also been recorded in Southern peninsula and Central India. Vertical distribution has been reported to up to 1300 m above sea level in Garhwal (Utteranchal) and 1100 m in Nilgiri Hills (Tamil Nadu).

### 1.4.2. Habit and habitats

The vector is crepuscular in its habit, inactive during daytime, and seeks shelter in cracks and crevices in the dark corners of houses of cattle sheds (Palit et al., 1996). In outdoor situations, it is found in caves, crevices, animal burrows, termite hills, tree holes etc. The sandflies are incapable of flying long distances and move by characteristic hopping movement. They have been detected up to a height of 2.74 m from the ground. It is found throughout the World inter tropical and temperate regions.

### 1.4.3. Seasonal prevalence

Studies conducted in endemic areas revealed that the vector density starts increasing from February onwards, with some decrease in May to June, followed by an increase with the advent of the monsoon. In Southern and Eastern India, with very mild cold season, *P. argentipes* is common throughout the year.

### 1.4.4. VL control strategies

The current control strategies for VL rely on reservoir and vector control, the use of insecticide-impregnated materials and active case detection and treatment (Boelaert *et al.*, 2000; Davies *et al.*, 2003) anti-leishmanial vaccines are still being developed.
1.4.4.1. Reservoir control

Dogs are the main reservoir of L. infantum in zoonotic VL. Despite evidence from experimental studies showing a decreased incidence of VL in both dogs and children following serological screening of dogs and killing of sero-positive animals (Ashford et al., 1998; Palatnik-de-Sousa et al., 2001), the efficiency and acceptability of this control strategy is increasingly being debated (Alvar et al., 1994; Tesh, 1995; Reithinger and Davies, 2002). Treating infected dogs is not an effective control strategy as relapses are frequent and dogs can regain infectivity weeks after treatment, despite being clinically cured (Alvar et al., 1994). Moreover, the widespread veterinary use of VL drugs might lead to resistance in parasites. A new control approach is the use of deltamethrine-treated collars, which reduced the risk of infection in dogs (by 54%) and children (by 43%) in a study conducted in Iran (Gavgani et al., 2002). Vaccination of dogs would nevertheless be the best strategy if an efficacious vaccine can be developed.

1.4.4.2. Vector control

Sandflies are susceptible to the same insecticides as Anopheles mosquitoes, the malaria vector. Residual insecticide spraying of houses and animal shelters was shown to be efficacious in India (Kaul et al., 1994), where the vector (Phlebotomus argentipes) is restricted to areas in and around the home. Following the large scale antimalarial insecticide (dichloro-diphenyl-trichloroethane (DDT)) spraying campaigns that were implemented in the 1950s, VL almost completely disappeared from the Indian subcontinent. Unfortunately, the disease quickly re-emerged when these spraying campaigns were discontinued. Resistance of P. argentipes to DDT remains limited, but has been reported in Bihar (Singh et al., 2001). In Sudan and other endemic countries in East Africa, transmission occurs mainly, but not exclusively (Hassan et al., 2004), outside villages, during shepherding for example. Indoor residual spraying for disease control is therefore unlikely to be as efficient in this region.

1.4.4.3. Insecticide-impregnated materials

The use of insecticide- treated bednets (ITNs) could concomitantly prevent VL and other vector-borne diseases, such as malaria and Japanese encephalitis. There is limited evidence that bednets provide protection against VL. Case-control studies conducted in Bangladesh and Nepal showed that sleeping under a non-impregnated bednet during the warm months was a protective factor against VL (Bern et al., 2000; Bern et al., 2005). Despite low usage, the mass distribution of ITNs in Sudan was accompanied by a 27%
reduction in the incidence of VL in an observational study (Ritmeijer et al., 2007). A large prospective randomized controlled trial testing the efficacy of long-lasting ITNs to prevent *L. donovani* infection and VL is underway in Nepal and India. Depending on the sleeping traditions of the population and the biting habits of the local vector, other insecticide-impregnated materials such as curtains and blankets should be evaluated for use in VL prevention, as some have been shown to provide efficient protection against cutaneous leishmaniasis (Reyburn et al., 2000; Kroeger et al., 2002).

### 1.5. Life cycle

During their complex life cycle, the single-celled parasites of the genus *Leishmania* are exposed to different extra and intracellular environments. These organisms are digenetic parasites with two basic life cycle stages: one extracellular stage within an invertebrate host (phlebotomine sand fly) and one intracellular stage within a vertebrate host. Thus, the parasites exist in two main morphological forms, amastigotes and promastigotes, which are found in vertebrate hosts and invertebrate hosts, respectively (Fig.1.3).
1.5.1. Stages in the Invertebrate Host (Promastigote)

The invertebrate hosts or vectors are small insects of the order Diptera, belonging to the subfamily Phlebotominae. They are commonly called phlebotomine sand flies. Of the six genera described, only two are of medical importance: Phlebotomus of the 'Old World', divided into 12 subgenera, and Lutzomyia of the 'New World', divided into 25 subgenera and species groups. All known vectors of the leishmaniases are species of these two genera. Among the 500 known phlebotomine species, only 31 have been positively identified as vectors of pathogenic species of Leishmania and 43 as probable vectors (Volf et al., 1994; Killick-Kendrick, 1999). The sand fly species involved in the transmission of Leishmania
vary from one geographical region to another but also depend on the species of *Leishmania* (Volf *et al.*, 1994; Killick-Kendrick, 1999). Like mosquitoes, the female needs a blood meal for egg development and only the female is haematophagous. Some phlebotomine species can support the growth of only those species of *Leishmania* with which they are infected in nature, such as *Phlebotomus papatasi* and *P. sergenti*; these species are considered to be restricted vectors (Kamhawi *et al.*, 2000). By contrast, other phlebotomine species such as *Lutzomyia longipalpis* and *Phlebotomus argentipes* are permissive vectors since they are able to develop mature transmissible infections when infected with several *Leishmania* species (Kamhawi *et al.*, 2000; Sadlova *et al.*, 2003; Warner *et al.*, 2004). Within the intermediate host, *Leishmania* develops as promastigote forms with a cell body measuring 5-20 x 1-4 mm, elongated motile extracellular stages possessing a prominent free flagellum up to 20 mm-long. Nevertheless, a variety of different promastigote forms have been distinguished on morphological grounds (Bates and Rogers, 2004).

### 1.5.2. Stages in the Vertebrate Host (Amastigote)

In the vertebrate host, the parasite evolves into an amastigote form. Amastigotes are ovoid (2.5–5 mm diameter), non-motile, intracellular stages. They do not have a free flagellum and are located in the parasitophorous vacuoles of the host’s macrophages.

In both developmental forms, the flagellum emerges from a flagellar pocket and in the amastigote form, it is almost completely restricted to it, so it is only observed by electron microscopy.

Infection begins when an infected female sand fly takes a blood meal from a healthy human host. Following inoculation into the skin by the sand fly bite, the infective flagellated metacyclic promastigotes are ultimately ingested by macrophages via receptor-mediated endocytosis (Chang *et al.*, 1990), transforms into amastigotes, and multiplies by binary fission. The infected macrophage eventually bursts and the released parasites are able to infect new phagocytic cells. When the infected host is bitten by another female sand fly, at the time of infective blood meal, the amastigotes in the gut of sandfly, due to change in temperature and other conditions, develop into flagellated promastigotes and the life cycle continues.

### 1.6. Current options for treatment

Despite the considerable progress made in the study of the biochemistry, physiology and molecular biology of *Leishmania* parasites, the absence of effective vaccines and vector
control programs, makes chemotherapy the only tool against leishmaniasis. The current situation for the chemotherapy of leishmaniasis is more promising than it has been for several years with both new drugs and new formulations of old drugs either recently approved or on clinical trial (Guerin et al., 2002; Croft and Coombs, 2003; Croft et al., 2006). The drugs available for treatment of Leishmania infections are as under-

1.6.1. Antimonials

Antimony (Sb) was acclaimed as one of the “Seven Wonders” of the World published in a treatise in Leipzig in 1604. It was introduced by Paracelsus as a general panacea in the 16th century, occasionally banned and often argued over for another three centuries, the modern era of usage began in 1905 when Plimmer and Thompson showed the activities of sodium and potassium tartrate (PTA) against trypanosomes in rats and subsequently in the treatment of human trypanosomiasis in Africa. The first published records of use of these trivalent antimonials for treatment were by Macado and Vianna in 1913 for CL, by di Cristina and Cariona in Sicily and Rogers in India in 1915 for VL (Schmidt and Peter, 1938). The discovery and synthesis of urea stibamine in 1920, by Prof. U.N. Brahmachari, was the most significant milestone in the control of VL. The death of Sir Brahmachari in 1946 and the dramatic decline in the incidence of Kala-azar due to destruction of Phlebotomus argentipes, the common vector of L. donovani in India, by DDT used to malaria destruction programme after World War II resulted in misuse of urea stibamine. Its success became overshadowed and paved the way of the development of the less toxic pentavalent antimonials. In 1937 Brahmachari, Schmidt, Kikuth and others led to the synthesis of antimony gluconate (Solustibosan) (Kikuth and Schmidt, 1937) and sodium stibogluconate (Pentostam) in 1945 (Goodwin, 1995). Another carbohydrate complex, meglumine antimoniate (Glucantime®, Aventis) soon followed. Therefore, important antimonial drugs are tartar emetic, sodium stibogluconate, meglumine antimoniate and derivatives of stibanilic acid such as stibamines, stibacetin, ethyl stibamine and urea stibamine. Out of these drugs sodium stibogluconate and meglumine antimoniate were found to be highly effective. Although, the structure of stibogluconate is still unknown despite its use for 50 years as the first line drug for VL. Treatment of leishmaniasis still relies to a large extent on pentavalent antimony compounds such as meglumine antimoniate (Glucantime®, Aventis) and sodium stibogluconate (Pentostam®, GSK) (Herwaldt, 1999; Guerin et al., 2002).
(a) Sodium stibogluconate- This is the safest and most potent drug among the pentavalent antimonials and has been widely used for nearly a half century (Cook, 1990) to treat VL rather than cutaneous and mucocutaneous leishmaniasis (Thakur et al., 1988) finding wider usage in India, China, Middle East, Africa and North America. The WHO (WHO, 1990) has recommended a daily dose of $\text{Sb}^\text{V}$ $20 \text{ mg/kg}$ for 30 days. In India, up to 60% VL patients in Bihar state do not respond to pentavalent antimonials (Croft and Coombs, 2003). An altered regimen of $20 \text{ mg/kg}$ per day has been recommended for more than 40 days (Thakur et al., 1988).

Bryceson, (1987) proved that $\text{Sb}^\text{V}$ is more effective if given more frequently than once daily and his data was corroborated by data of Zijlstra et al. (1991) who showed that $\text{Sb}^\text{V}$ at $10 \text{ mg/kg}$ every 12h for 15 d was better than $20 \text{ mg/kg}$ per day for 30 days. Chulay et al. (1983) also showed that $10 \text{ mg/kg}$ every 8h for 10 days was more effective than $20 \text{ mg/kg}$ per day for 30 days.

(b) Meglumine antimoniate- This is also a drug of choice for treating leishmaniasis and has been widely used in Latin America at the recommended adult dose of 17-28 mg/kg/d for 10 to 20 days.

Although pentavalent antimony compounds have proven to be very effective, drug use is often limited in patients due to toxic side effects such as nausea, abdominal pain, chemical pancreatitis, renal toxicity and, especially worrisome, electrocardiographic abnormalities (Guerin et al., 2002). The cardiotoxicity of pentavalent antimony compounds, which may include inversion of the ST-segment on the electrocardiogram, QTc prolongation, torsade de pointes arrhythmias (TdP) and sudden cardiac arrest (Chulay et al., 1985; Ortega-Carnicer et al., 1997; Thakur, 1998; Berhe et al., 2001) (Cesur et al., 2002), severely limits prolonged treatment courses in patients in particular when high concentrations are indicated to combat and overcome resistance.

The exorbitant cost of brand formulations of $\text{Sb}^\text{V}$ prompted Medicins Sans Frontieres to commission three studies in Sudan, Kenya and Ethiopia to compare the efficacy in VL of the generic sodium antimony gluconate (SAG) (Albert David, Kolkata India, costs US $13 per patient) vs. branded SAG (Pentostam, Glaxo-Wellcome, UK, costs US $200 per patient) (Veeken and Ritmeijer, 2006). It was conclusively proven that no significant difference existed between the two formulations as generic SAG was equally effective in terms of efficacy and safety in all forms of leishmaniasis and importantly,
achievable at a substantially lower cost. However, caution must be exercised before using SbV from new manufacturers as bad batches caused fatal cardiotoxicity (Sundar et al., 1998). In two reports from India and Nepal, high incidence of fatal cardiotoxicity was reported with use of antimony made from an unknown manufacturer (Sundar et al., 1998; Rijal et al., 2003).

Post kala-azar dermal leishmaniasis (PKDL), a dermatological manifestation generally following VL infection occurs predominantly in India and Sudan. Although in both L. donovani is the causative organism, Indian PKDL requires prolonged treatment (>120 days) (Thakur and Kumar, 1990) whereas for the Sudanese variety, two months treatment is considered adequate (Kamil et al., 2003).

The precise mechanism of action remains unknown, although antimonial compounds have been used in the treatment of leishmaniasis for at least 100 years. Interpretation of some of the earlier reports on mode of action and drug sensitivity to antimonials is complicated by the fact that liquid formulations of sodium stibogluconate contain the preservative m-chlorocresol, itself a potent antileishmanial agent (Brown et al., 1993). However, Goodwin and Page (1943) were the first to propose that pentavalent antimony SbV might act as a prodrug that has to be converted into active, trivalent SbIII which is thought to interfere with thiol-dependent redox systems of Leishmania parasites (Kellinghaus et al., 2004). The details of the activation mechanism and the exact site of this conversion of SbV to SbIII are still unclear. However, several studies have reported that axenic amastigotes (i.e., culture turned in the absence of macrophages) are susceptible to SbV, whereas promastigotes are not, suggesting that some stage specific reduction occurs in this life cycle stage (Ephros et al., 1999; Goyard et al., 2003). Biochemical studies over the past two decades have indicated a number of potential targets for pentavalent antimonials; glycolysis in particular inhibition of ADP phosphorylation (Berman et al., 1985), DNA I topoisomerase (Chakraborty and Majumder, 1988; Lucumi et al., 1998), inhibition of fatty acid beta-oxidation (Berman et al., 1989) and trypanothione (Mukhopadhyay et al., 1996; Legare et al., 1997). Recent studies have shown that both SbIII and SbV mediate DNA fragmentation in Leishmania species, suggesting that antimony kills the parasite by a process reminiscent of apoptosis (Sereno et al., 2001a; Lee et al., 2002; Sudhandiran and Shaha, 2003).
1.6.2. Pentamidine

Pentamidine, an aromatic diamidine, as the isethionate salt (Pentacarinat) and previously as the methylsulphonate salt (Lomidine), have been used as alternative treatments for VL, CL and DCL since 1952. As a second line drug for antimony resistant cases it has proved useful in India and Kenya (Thakur et al., 2001). The efficacy of pentamidine against antimony refractory infections has decreased over the years (Jha et al., 1991; Sundar, 2001). Its use has been largely abandoned in India where pentamidine failures are common, but it continues to be used alone or in combination with other drugs in other countries (Basselin et al., 1997a; Heath et al., 2001). Although use of pentamidine for the treatment of CL was revisited in the 1990s, with clinical trials for treatment of New World CL, this drug is not a widely used antileishmanial drug. Pentamidine was shown to be highly effective against CL in Colombia in a short course low dose regimen (Soto et al., 1994). For VL there are some indication of emergence of resistance have been reported. Pentamidine resistant promastigote clones of L. donovani and L. amazonensis (Basselin et al., 2002). Although, specific transporters for pentamidine uptake have been characterized and might have a role in resistance (Bray et al., 2003; Coelho et al., 2003). Coelho et al. (2003) have reported pentamidine resistant gene belonging to the P-glycoprotein (PGP)/MRP ATP-binding cassette (ABC) transporter superfamily, named pentamidine resistance protein 1 (PRP1), might have a role in pentamidine resistance. Others data have shown the accumulation of more pentamidine in the wild type Leishmania mitochondrion than the resistant cells, is being of importance for more drug efflux (Basselin et al., 2002).

The antileishmanial mechanism of action of pentamidine remains incompletely understood (Bray et al., 2003), and there are probably multiple targets, including polyamine biosynthesis (Basselin et al., 1997b), DNA minor groove binding and mitochondrial inner membrane potential (Vercesi and Docampo, 1992). Toxicity has always been a limitation on use with reports of hypoglycaemia, diabetes, nephrotoxicity, tachycardia, pain at site of injection (Jha, 1983; Soto et al., 1994). Pentamidine is still used for treatment of haemolymphatic stage of human African trypanosomiasis (Pepin and Milord, 1994) and, in combination with sulfamethoxazole, for Pneumocystis carinii pneumonitis (PCP) in AIDS patients.

1.6.3. Amphotericin B and its lipid formulations

Amphotericin B (Amp B), a macrolide polyene antibiotic isolated from Streptomyces nodosus, was first shown to have antileishmanial activity in the early 1960's...
and was soon used in the treatment of mucocutaneous leishmaniasis (Sampaio et al., 1960; Fuchs et al., 2003). Amp B is predominantly used as an antifungal drug, specifically for treating systemic mycoses. The selective activity of Amp B against fungi and Leishmania is due to the higher affinity of the drug for 24-substituted sterols, found in the plasma membrane of these eukaryotic microorganisms, over cholesterol in the plasma membranes of mammalian cells. Amp B in a dose of 0.75-1 mg/kg for 15 to 20 infusions either daily or on alternate days has consistently cured about 97 per cent of VL patients (Sundar and Rai, 2002). Although Amp B has long been considered as an alternative treatment for MCL and VL (WHO, 1996), its use has been restricted by infusion related and delayed toxic side effects, in particular cardiotoxicity and nephrotoxicity and even deaths (Khoo et al., 1994).

However, antileishmanial chemotherapy has benefited from the development of lipid-associated formulations of Amp B. In these formulations, deoxycholate has been replaced by other lipids that mask toxicity of Amp B and facilitate its preferential uptake by reticuloendothelial cells, thus achieving targeted drug delivery to the parasite resulting in increasing efficacy and reduced toxicity and an extended plasma half-life in comparison to the parent drug. Three such lipid associated formulations are:

(i) Liposomal amphotericin B (AmBisome; Gilead Sciences, Foster City, CA, USA)
(ii) Amphotericin B lipid complex (Abelcet; The Liposome Co, Princeton, NJ, USA)
(iii) Amphotericin B colloidal dispersion (Amphocil; Sequus Pharmaceutical; Menlo Park, USA).

These formulations have been successfully used in clinical trials for VL and/or MCL while, AmBisome was proven quite effective (Berman et al., 1987). In Bihar (India) a comparative study of parental Amp B (1 mg/kg x 30, at alternative days) with AmBisome and Abelcet (both at a dose of 2 mg/kg/day for 5 days) showed the similar overall cure rates but, the lipid formulations had an upper edge as they produced distinctly lower toxicities. Liposomal Amp B is considered by many experts as the best existing drug against VL, and is used as first-line treatment in Europe and the United States. However, in terms of cost factor, the lipid formulations were two times more expensive than Amp B (Sundar et al., 2004). Until recently, its use in developing countries was precluded by its high market price (US$2,800 per treatment) (Gradoni et al., 1993; Bern et al., 2006). This situation might change as the World Health Organization (WHO) announced a drastic price reduction in
May 2007, with the cost of an average course for the public health sector in VL-endemic countries reduced to US$200.

There have been two small inconclusive studies on the emergence of Amp B resistance in *L. infantum*/HIV-infected cases in France. One study failed to find a change in sensitivity in promastigotes derived from isolates taken before and after the treatment of one patient (Durand et al., 1998). In contrast, a decrease in sensitivity was observed in isolates taken over several relapses from another patient (Di Giorgio et al., 1999). There has been increased use of Amp B for visceral leishmaniasis, in both the deoxycholate (Thakur et al., 1998; Sundar et al., 2002b) and lipid formulations (Berman et al., 1998; Sundar et al., 2003), following failure of antimonial treatment and in HIV/VL coinfection cases. With the increasing use of amphotericin B in lipid formulations that have longer half-lives, the possibility of resistance cannot be ignored.

1.6.4. **Paromomycin (aminosidine)**

Paromomycin (PM), an aminoglycoside antibiotic, was originally identified as an antileishmanial in the 1960s and has been used for the treatment of VL in parenteral formulation in phase III and CL in both topical and parenteral formulations. Although development of the parenteral formulation of PM, a drug with poor oral bioavailability, for VL has been slow, several Phase 2 trials in India and Kenya have been promising, with 90% of patients cured of VL.

There are also encouraging findings on the use of PM as a topical treatment for CL. Elon and colleagues in 1984 have reported that a topical formulation containing 15% PM and 12% methyl benzethonium chloride (a skin penetrating agent) was effective against experimental CL led to clinical trials. Hydrophillic formulation of PM was found more effective than hydrophobic formulation (Goncalves et al., 2005). Combinations of paromomycin with antimonials have been effective against VL (Nuzum et al., 1995; Thakur et al., 2000) with the potential shortening of treatment. It acts by interfering with the mitochondrial activity and inhibiting cell respiration and lowering the electric potential difference across the mitochondrial membranes (Maarouf et al., 1998). Some studies indicated that binding to ribosomal units might be involved as paromomycin promoted subunit dissociation of both cytoplasmic and mitochondrial ribosomes (Maarouf et al., 1998).
The results from initial studies in India and Africa were promising (Chunge et al., 1990; Jha et al., 1998), but the original manufacturer abandoned production. The results of Phase III trials recently conducted in India showed excellent efficacy and safety. No nephrotoxicity was observed; reversible high-tone ototoxicity (damage to the inner ear) was found in 2% of patients and 1.8% of patients showed a significant increase (> fivefold) in hepatic transaminases (Sundar et al., 2007). Mild injection pain was reported by over 50% of patients. Paromomycin was registered in India in August 2006. In East Africa, paromomycin is currently being evaluated in mono- and combination (with sodium stibogluconate) therapy. Other advantages of paromomycin include the fact that it is active against a wide variety of pathogens, including bacteria, and its low cost (US$5–10 per treatment).

Resistance to aminoglycosides in bacteria is well known and has been characterized in relation to decreased uptake in gram-negative pathogens, alteration of the ribosomal binding, and modification of amino groups or hydroxyl groups by inactivating N-acetyltransferases, O-phosphotransferases, or O-nucleotidyl transferases (Davies and Wright, 1997). Paromomycin has had limited use in the treatment of visceral leishmaniasis, and there is no clinical resistance in this form of the disease have been reported. However, it has been used more extensively for the treatment of cutaneous disease. So far, there has been only one report suggesting resistance could develop. Following a 60-day parenteral course for treatment of two *L. aethiopica* cases, isolates taken from relapse patients were three- to fivefold less sensitive to the drug after treatment than isolates taken before treatment in an amastigote-macrophage assay (Teklemariam et al., 1994). In studies on selected populations of promastigotes, resistance was related to decreased drug uptake in *L. donovani* (Maarouf et al., 1998). In the analysis of small-subunit rRNA and DNA of a paromomycin-resistant *L. tropica*, small-subunit rRNA was eightfold more resistant to paromomycin and had a low level of cross-resistance to other aminoglycosides, showed no change in the sequence of the binding site. Monitoring of resistance could be of importance if paromomycin formulations are introduced as a first line treatment (Croft et al., 2006).

**1.6.5. Allopurinol and purine analogues**

The antileishmanial activity of the purine analogue allopurinol was identified over 30 years ago and, because it had oral bioavailability and was widely used for other clinical indications, it entered clinical trials for VL and CL. All parasitic protozoa studied to date are unable to synthesize purines *de novo* and, therefore, rely upon uptake and salvage of
preformed purines for survival. Allopurinol is used as a substrate by various enzymes of the purine salvage pathway of trypanosomatids, and is selectively incorporated into nucleotide intermediates and nucleic acids in the parasite. In recent years, allopurinol has been considered as part of a maintenance therapy for canine leishmaniasis, against which it has suppressive activity (Koutinas et al., 2001). Clinical trials of allopurinol in combination with other drugs have shown more promising results.

1.6.6. Sitamaquine

Sitamaquine, an orally active 8-aminoquinoline analog (8-aminoquinoline (8-[6-(diethylamino) hexyl] amino)-6-methoxy-4-methylquinoline), was originally developed as WR6026 by the Walter Reed Army Institute in collaboration with Glaxo Smith Kline. Phase II studies were conducted in Brazil, Kenya and India and obtained cure rates ranging from 27% to 87%, but there were also several cases of serious renal adverse events (Dietze et al., 2001; Jha et al., 2005; Wasunna et al., 2005). Phase IIb and III studies are ongoing and planned, respectively, in India. The drug has broad-spectrum antiprotozoal activity (Yeates, 2002)) but with limited clinical use and no reported resistance. The cure rate of sitamaquine for VL in clinical study showed 50-67 per cent at a dose of 1-2 mg/kg/day for 28 days (Sherwood et al., 1994), and increasing the dose to 2.5 mg/kg/day resulted in decreased efficacy concomitant with enhanced adverse effects such as nephropathy and methaemoglobinaemia (Dietze et al., 2001). In a multicentric phase II clinical trial in India, sitamaquine demonstrated excellent antileishmanial activity (overall 87%) at a daily dose of 1.5 – 2.0 or 2.5 mg/kg for 28 days (Jha et al., 2005). In a phase II clinical trial of sitamaquine dose responding study conducted in Kenyan patients with VL caused by L. donovani. Patients received sitamaquine daily for 28 days at one of four doses: 1.75, 2.0, 2.5, or 3.0 mg/kg/day and cure was achieved from 83-92%. The most frequent adverse events during active treatment were abdominal pain and headache and at higher dosage of 2.5 mg/kg/day and 3.0 mg/kg/day severe renal adverse events have been reported (Wasunna et al., 2005). 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 (Kinnamon 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. However,
more studies are needed to evaluate some of the safety issues, as this drug appears to have clinical efficacy that warrants further development.

1.6.7. Azoles

Azole such as ketoconazole, itraconazole, fluconazole, as antileishmanial agents has also been studied (Bahamdan et al., 1997; Salmanpour et al., 2001; Seth et al., 2002). The mechanism of action of ketoconazole against Leishmania promastigotes is the same as for Candida albicans, i.e., interference with membrane permeability (cytochrome P-450), secondary to loss of desmethyl sterols and accumulation of 14α-methyl sterols. These sterols have a detrimental effect on the membrane permeability and hence on the viability of the organism. Thus, the working hypothesis is that the accumulation of 14-methylsterols consequently leads to an alteration in the membrane fluidity and permeability.

The azole compounds have inevitably weaker action than amphotericin because they act in the synthesis of ergosterol by inhibiting the demethylation of lanosterol (Urbina, 1997). These have not been consistently effective when used alone for the treatment of VL (Sundar et al., 1996), and are employed in combination with allopurinol or pentavalent antimonials. Fluconazole has, however, been successfully used for CL infections caused by L. major (Seth et al., 2002).

1.6.8. Miltefosine

Perhaps the most significant recent advancement in the antileishmanials discovery has been the effective oral treatment of VL by using miltefosine, an alkylphosphocholine, originally developed as an anticancer drug (Croft et al., 2003). The antileishmanial activity of miltefosine was initially discovered in the mid 1980s, after a phase 3 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., 2002a). From the pilot study Sundar et al. (2006) have reported that miltefosine is a good oral agent for the treatment of Indian VL, both for the treatment of naïve and Sb" unresponsive patients. Miltefosine was registered for phase IV in India in March, 2002 for oral treatment of VL and in Colombia for CL in 2005 for phase III clinical trials and has now been introduced into the market in India (Croft et al., 2006). The major limitation of miltefosine is teratogenicity and this excludes its use in women of child-bearing age. The long half-life of miltefosine (2–3 weeks) and its narrow therapeutic index might favour the emergence of resistant mutants. Due to the recent introduction of miltefosine in the field, not too many clinically resistant parasites have been
reported. Before entering in to the market or in to control programmes, preliminary data from a phase IV trial in India involving domiciliary treatment with miltefosine and weekly supervision suggests doubling of the relapse rate; this provides warning that drug resistance could develop quickly and plans are required to prevent it (Croft et al., 2006). However, resistance can be easily induced in vitro and some of the resulting mutants have been characterized (Seifert et al., 2003). *L. donovani* promastigotes having mutation in putative miltefosine transporter were shown to be responsible for the reduced uptake of miltefosine. The potential relevance of these observations needs to be extended to miltefosine resistant amastigotes before clinical implications to be made. Combination therapy should be considered, to delay the emergence of miltefosine resistance, particularly in anthroponotic foci where resistance could quickly spread (Bryceson, 2001). The price of the drug is currently under discussion, but to be useful in endemic countries it should be in the range of, and preferably lower, than that of current first-line treatment options. While, Miltefosine has been registered in India since 2002 and so far it has only been available on the private market, at a retail cost of US$125–200 per treatment course (Sundar and Murray, 2005; Bhattacharya et al., 2007). The drug has a long terminal half-life (~7 years). The fact that Miltefosine is administered orally and produces early symptomatic improvement predisposes it for incomplete treatment unless the treatment is strictly supervised. All these factors could encourage development of resistance to the drug and premature end of this very important arsenal against *Leishmania* unless it is handled and used judiciously.

Miltefosine received approval by the Indian regulatory authorities in 2002 and in Germany in 2004. In 2005 it received the first approval for cutaneous leishmaniasis in Colombia. Miltefosine is also currently being investigated as treatment for mucocutaneous leishmaniasis caused by *L. braziliensis* in Colombia, (Soto and Toledo, 2007), and preliminary results are very promising. It is now registered in many countries and is the first orally administered breakthrough therapy for visceral and cutaneous leishmaniasis (Jha et al., 1999; More et al., 2003). In October 2006 it received orphan drug status from the US Food and Drug administration. The drug is generally better tolerated than other drugs.

1.7. Screening model

The primary pre-requisite for any drug development programme lies on the availability of a suitable in vitro and or in vivo experimental system. There are several in vitro and in vivo systems, each with specific characteristics, available for lead optimization have been summarized as under-
1.7.1. *In vitro system*

The *in vitro* system may be of potential use for compounds, which have direct lethal action on parasite but the compounds, which are effective through their metabolites, or their action is mediated through host defense system will not show any action. Hence, there remains a glitch on the acceptability of *in vitro* results. However, *in vitro* drug testing has many advantages, e.g.

(a) The parasites from a few animals are sufficient to test many compounds
(b) The requirement of test compound is very minute
(c) The turnover of screening results are quick and
(d) The results are consistent.

However, the *in vitro* testing at times may not be transferable to *in vivo* situation particularly for compounds, which undergo biotransformation before exerting anti-parasitic action. Besides, such compounds which act through immune system, did not show activity in *vitro*, though may be highly potential in animals.

Fortunately, in leishmaniasis very close correlation exists between the *in vitro* and *in vivo* results (Bhatnagar *et al.*, 1989), because the test parasite is the disease-producing organism in human (amastigote) and these are maintained *in vitro* as axenic amastigotes and in macrophage culture presenting a semi-*in vivo* condition.

In 1987, Croft outlined the requirements for an *in vitro* assay which include use of-

(i) mammalian stage of the parasite,
(ii) a dividing population,
(iii) quantifiable and reproducible measures of drug activity, and
(iv) activity of standard drugs in concentrations achievable in serum/tissues.

Recently, assay design has focused on features that make the system adaptable to high throughput screening (HTS), with additional requirements of (i) small amounts of compound (less than 1 mg), (ii) quick throughput, and (iii) low cost of tests.

Whatsoever, good *in vitro* system is used; the test results need to be verified in animals. Screening of compounds for leishmanicidal activity can be performed with promastigotes and amastigotes of the parasite cultured under axenic conditions, as discussed under-

I. **With promastigotes**

The simplest model to be utilize is the one in which the promastigotes multiply in cell free media (Neal, 1984). For drug testing promastigotes are diluted to a concentration of $1.0-2.0 \times 10^6$ per ml of cultivation medium and the drugs in appropriate concentrations are
added to the experimental culture. The inhibition of promastigote multiplication is assessed after approximately 3 days, during which the control organism multiply 3-6 times. The technique is simple and easily applicable. However, the metabolism and ecology of promastigote differ so widely from those of amastigote (target form) that screening data obtained from \textit{in-vitro} test with promastigote have very little value in animals (Peters et al., 1983). The other conditions which reduce leishmanicidal action \textit{in vitro} are the lower temperature (24 °C) at which the culture normally grows, as opposed to the \textit{in vivo} temperature of 37 °C. The promastigote in culture at 37 °C will survive but not multiply. Further, the promastigote culture represents an artificial situation and is of little or no value for drug screening. Due to these problems, the use of promastigote for drug testing has been abandoned.

However, Jackson et al. (1989) have developed an \textit{in vitro} micro test for drug sensitivity, which is quantitative, rapid and readily applicable to parasites isolated from all major forms of human leishmaniasis as it uses promastigotes converted from amastigotes \textit{in vitro}. The test is in a serum free chemically modified medium containing 120-μg protein/ml. They have claimed that \textit{Leishmania} sensitivity to pentavalent antimonials is detectable at the levels below concentrations achievable in patient’s sera.

\section*{II. With amastigotes}

In leishmaniasis, the amastigotes live and multiply in the parasitophorous vacuole of the phagolysosomes. The discovery of maintenance of infected macrophage \textit{in vitro} solved many problems of \textit{in vitro} drug testing against amastigotes. This system (macrophage-amastigote) has received wide recognition since the system provides more or less \textit{in vivo} environment to the parasites.

\subsection*{II. 1. In tumor macrophages}

The amastigotes have been grown in tumor macrophages \textit{in vitro}. Mattock and Peters (1975) have extensively studied the effect of drugs on amastigotes of \textit{L. donovani} in dog sarcoma cells. The disadvantage of this model is that tumor cells are self-multiplying while macrophages are non dividing. Thus, the biochemical environment of host cells in the two cases is different and as drug interferes with biochemical pathways, the interaction of drug in tumor macrophages \textit{in vitro} might differ from \textit{in vivo} (Berman et al., 1985).
II. 2. Mouse peritoneal macrophages

For in vitro drug testing, Peters et al. (1980) employed infected mouse peritoneal macrophages, which were also used by Neal and Mathew (1982), Neal and Croft (1984). Though, the mouse peritoneal macrophages are well suited for amastigote culture, these cannot be considered as ideal since the properties of rodent macrophages may not correspond to human reticulo-endothelial cells and the therapeutic results are likely to vary.

II. 3. Human monocyte

Human monocytes were used on the assumption that environment within these might mimic the environment of human patients (Haberman et al., 1979). In this system, the human mononuclear cells isolated from peripheral blood, are cultured in plastic wells for 6 days. During this time, half of the monocytes have adhered to the plastic bottom of the chambers and have enlarged into macrophages. These macrophages are infected with Leishmania parasites and then the chemotherapeutic trial begins. THP-1, U937, HL-60 monocytic cell lines have been used in drug assays (Gebre-Hiwot et al., 1992). The practical disadvantage with this system is that it requires a large amount of blood and needs longer period for culture prior to experimentation (Zil'berman and Koromyslov, 1982). Otherwise, theoretically this model is most appropriate because it resembles best with the clinically infected macrophages.

III. With axenic amastigotes

Axenic amastigote cultures offer different opportunities and dimensions to in vitro drug screening and have been used for L. mexicana (Bates, 1994; Callahan et al., 1997), L. braziliensis (Balanco et al., 1998), L. donovani (Sereno and Lemesre, 1997; Ephros et al., 1999) and L. infantum (Sereno et al., 2000). Differences in drug sensitivity between axenic L. donovani amastigotes and intracellular amastigotes have been observed (Ephros et al., 1999).

1.7.1.1. Viability assay

There are various test systems for viability assessment of parasites in presence or absence of drugs, some of which are briefly being described below.

a) Microscopic observation

Although the current drug screening has been revolutionized with the advancement of molecular and biochemical sciences but the present screening has remained with for
Giemsa staining and microscopic counting of parasites (Berman, 1984b; Callahan et al., 1997). This method is traditional, labor intensive and time-consuming.

b) MTT reduction assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann (1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and generate reducing equivalents such as NADH and NADPH, form a dark blue formazan crystals. The resulting dark blue formazan crystal is largely impermeable to cell membranes, thus accumulates within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals, which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer (ELISA reader).

The metabolic reduction of soluble tetrazolium salts to insoluble colored formazans has been exploited for many years for histochemical localization of enzyme activities (Altman, 1976). In one of the earliest efforts to develop a practical in vitro drug sensitivity test, Black and Speer (1953) utilized a tetrazolium/formazan method to assess inhibition of dehydrogenase activity by cancer chemotherapeutic drugs in slices of excised tissue. As an in situ vital staining process, this phenomenon has also been used for identifying viable colonies of mammalian cells in soft agar culture (Schaeffer and Friend, 1976) and for facilitating in vitro drug sensitivity assays with human tumor cell populations in primary culture (Alley and Lieber, 1984). Mosmann (1983) described a tetrazolium-based assay which allowed rapid measurement of growth of lymphoid cell populations and their response to lymphokines. Alley et al. (1988), Carmichael et al. (1987) described modifications of Mosmann's procedure for in vitro assay of tumor cell response to chemotherapeutic agents. Scudiero et al. (1988) found that DMSO is required for solubilization of MTT-formazan generated by cellular reduction of the MTT tetrazolium reagent. This step is not only laborious, but also may risk exposure of laboratory personnel to large quantities of potentially hazardous solutions in DMSO. Frequent DMSO exposure also produces deleterious effects upon some laboratory equipment. To overcome the drawback, a series of new tetrazolium (XTT) salts have been developed which, upon metabolic reduction by viable cells, yield aqueous-soluble formazans (Paul et al., 1988).
Nakashima et al. (1989) have used this method in cell plaque assay for human immunodeficiency virus (HIV) and Phelouzat et al. (1993) for viability assay of Leishmania promastigote. Standard anti-leishmanial drugs were tested, with MTT assay, for their ability to inhibit the growth of intracellular amastigotes of L. aethiopica, L. donovani and L. infantum in the human leukemia monocyte THP-1 cell line (Gebre-Hiwot et al., 1992). Gebre-Hiwot and Frommel (1993) by using this assay investigated ergosterol inhibitors against L. donovani grown as intracellular amastigotes in the human leukaemia monocyte cell line, THP-1 and against promastigotes by Nakayama et al. (2007). Though this is laborious, time consuming, dangerous and it’s fate depends on live cell metabolism but, yet in use in various laboratories.

c) Alamar Blue oxidation-reduction assay

Resazurin is a phenoxazin-3-one dye known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor (Page et al., 1993). Alamar Blue is blue in its oxidized form. When reduced by bacteria or tissue culture cells, it changes to a bright pink color that can be measured at 570 nm in the visible range or in the fluorometric UV range at 590 nm. The oxidized blue state can only be read at 600 to 630 nm in the visible range, and it is not fluorometric. The transformation of non-fluorescent resazurin to fluorescent resorufin has been used as a fluorometric indicator for the determination of cell viability. Although it has been arbitrarily postulated as being reduced by mitochondrial enzymes (De Fries et al., 1995), recent studies suggest that other enzymes, such as diaphorases (Zalata et al., 1998) located both in the cytoplasm and in mitochondria, are mainly responsible for reduction of the dye. Thus, the decrease in resazurin reduction probably indicates an impairment of cellular metabolism, rather than specific mitochondrial dysfunction.

Resazurin has been used since the 1950s to assess bacterial or yeast contamination in biological fluids (Erb and Ehlers, 1950) and it is used to measure the viability of sperm by colorimetry (Carter et al., 1973). It has been commercialized since 1993 as Alamar blue dye (O’Brien et al., 2000) as a viability cell test. It has been assessed on different types of cells for its cytotoxicity reliability, i.e. fibroblasts (Voytik-Harbin et al., 1998), immortalized and cancer cell lines (Page et al., 1993; Nakayama et al., 1997), tumor necrosis factor-hypersensitive cells (Shahan et al., 1994), and for its cell proliferation reliability on mouse and human lymphocytes (Ahmed et al., 1994), and primary neuronal cell culture (White et
al., 1996). Viability measurement using resazurin is similar to or greater than traditional tetrazolium salts (MTT, XTT) and [³H] thymidine assay techniques (Larson et al., 1997). This system has been exploited susceptibility testing for gram-negative bacteria by (Baker et al., 1994), for yeasts by Pfaller and colleagues (1994), and for enterococci (vancomycin only) by Tenover et al. (1995) and Zabransky et al. (1995). Raz et al. (1997) have used this oxidation-reduction indicator in drug sensitivity assays with culture adapted bloodstream forms of Trypanosoma brucei gambiense and T. b. rhodesiense and have been exploited by several laboratories to measure cytotoxicity of compounds against the protozoan parasite like L. major (Mikus and Steverding, 2000), antibacterial diterpenes isolated from Premna schimperi and P. oligotricha against L. aethiopica (Habtemariam, 2003), aromatic dicationic compounds against L. infantum (Rosypal et al., 2007).

Resazurin assay is a simple and rapid test in which a 0.01 mg/ml (40 μmol/l approximately) solution is added to the medium and measured either by colorimetry or fluorometry. However, greater sensitivity is achieved using the fluorescent property. Resazurin is non-toxic to cells and does not need killing the cells to achieve measurement.

MTT and Alamar Blue have been used as the color indicator for drug screening in the Leishmania promastigote assay (Phelouzat et al., 1993; Mikus and Steverding, 2000). This metabolic dye reflects viability by cellular reduction of the dye to a fluorescent form, or conversion from blue to red. Thus, as a group, oxidants/antioxidants may interfere with the reaction and the assay can not be used for testing these compounds.

1.7.1.2. Enzyme assay

The enzyme assays have been devised to screen the lead compounds and are based on the ability of target cells to reduce specific substrate to its constituents that can be measured by means of spectrophotometer. Bodley and Shapiro (1995) have developed phosphatase activity for assessing the antiparasitic activity of compounds against axenically cultured, bloodstream-form trypanosomes, T. brucei and L. donovani promastigotes. They measured the phosphatase activity in surviving cells in buffer containing p-nitrophenyl phosphate. The phosphatase enzyme catabolizes the p-nitrophenyl phosphate into p-nitrophenol, which was measured at 405 nm on a microtiter plate reader.

The nitrate reductase assay is a drug susceptibility test, used for biochemical identification of mycobacterial species, detects the presence of nitrite with specific reagents, which produce a change in colour (Angeby et al., 2002).
Buckner et al. (1996) have developed a new drug screening method by utilizing *T. cruzi* cells that express the *Escherichia coli* β-galactosidase reporter gene (Seeber and Boothroyd, 1996). Transfected parasites catalyze a colorimetric reaction with chlorophenol red β-D-galactopyranoside as substrate. Parasite growth in the presence of drugs in microtiter plates was quantitated with an enzyme-linked immunosorbent assay reader. The drugs benznidazole, fluconazole, and amphotericin B were shown to inhibit the parasites at concentrations similar to those previously reported. They tested several compounds that are inhibitors of glyceraldehyde-3-phosphate dehydrogenase of the related organisms *L. mexicana* and *T. brucei*. The drawback of enzyme assays was observed with colored drugs, which can interfere the results and some compounds may alter the effects of enzyme or vice-versa e.g. β- galactosidase (Buckner et al., 1996).

1.7.1.3. GFP fluorescence assay

GFP (green fluorescent protein) is a highly fluorescent and stable protein, which originates from the jellyfish *Aequorea victoria* (Prasher et al., 1992). GFP fluorescence is easily monitored using fluorescence-based equipment. The protein does not require substrates or cofactors to fluoresce, which makes quantification possible without the need for invading cells (Tsien, 1998). GFP-leishmanial fusion proteins have been synthesized for localization and trafficking analyses (Debrabant et al., 2000). This has been developed as a reporter gene for a variety of organisms including *Mycobacterium* (Dhandayuthapani et al., 1995) and for the *in vitro* screening of compounds against bacteria and mammalian cells, e.g., *Mycobacterium tuberculosis* and cytotoxicity against mammalian cells (Collins et al., 1998; Steff et al., 2001). Ha et al. (1996), and Misslitz et al. (2000) have developed monomeric GFP-expressing *Leishmania* parasites that do not have sufficient intensity of fluorescence for spectrophotometric measurement and the tested compounds needs to be determined by quantifying the transfectants microscopically or by flow cytometry (Kamau et al., 2001; Plock et al., 2001). Subsequently, Chan et al. (2003) have developed a spectrophotometric assay for the protozoan parasite *L. amazonensis* by using multimeric (four monomers) green fluorescent protein (GFP), generating brightness, which are measurable in 96-well microtiter plates. They claimed the feasibility of this assay for primary drug testing. Later, Singh and Dube (2004) introduced GFP marker into field strains of *L. donovani* promastigotes. By using these GFP-transfected parasites they have developed *in vitro* screening system, which is being used for testing of anti-leishmanial
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drugs by flowcytometry (Dube et al., 2005). However, they could not develop the assay, which would be measured in 96-well microtiter plates.

Although, GFP reporter gene technology has been used as a convenient marker in eukaryotic organisms (Chalfie et al., 1994) and for primary drug screening for trypanosomes and Leishmania parasites, GFP requires molecular oxygen to catalyze dehydrogenation of the chromophore (p-hydroxybenzylidine-imidazolidinone) to its fluorescent form (Heim et al., 1994; Cubitt et al., 1995). This may be a problem for GFP use in systems in which the amount of molecular oxygen is limited. Other problems are the use of flowcytometry for result measurement, and this system could not be developed for HTS due to the limitation of the low level of fluorescence to be detected by fluorimeter (Ashutosh et al., 2005).

1.7.1.4. Luciferase assay

The luciferase reporter gene technology is being widely used to monitor cell growth and proliferation under in vitro culture systems and to monitor the cellular events associated with gene expression (Welsh and Kay, 1997) and signal transduction. The use of firefly luciferase reporter genes in a number of intracellular microorganisms including Mycobacterium tuberculosis (Jacobs et al., 1993) has facilitated antimicrobial drug testing and discovery. The firefly luciferase (Gould and Subramani, 1988) represents one of the most efficient biological reporter molecules, which allow monitoring host-microbe interactions (Valdivia and Falkow, 1997), rapid testing of cellular viability, and thus is most suitable for biological screening. The method is rapid, very sensitive, and highly reproducible and does not require any very expensive specialized instrument or training the only drawback of this system is the use of expensive substrate and lysis of cells to detect the signal.

1.7.2. In vivo system

Animal models enable drug activity to be determined in relation to absorption (route of administration), distribution (different sites of infection), metabolism (pro-drugs, immunomodulators), and excretion and to give an early indication of the toxicity. The aim of using the animal model is to find a drug that can be administered orally, be effective in a short course (less than 10 days) and have no indication of toxicity at the highest doses tested (100 mg/kg). Though efficacy trials in animals are adequately reliable and the conclusions drawn from them are frequently transferable to target host while, the in vivo trails are often
difficult to scale, labor intensive, expensive, lengthy. Several animal models for leishmaniasis have been developed, each with specific features, but none accurately reproduces the clinical situation. A summary of the available experimental models is briefly described below.

1.7.2.1. The mouse model

Mice are susceptible to most strains and species of Leishmania in both non-cure and self-cure models. For visceral leishmaniasis inbred strains of mice are widely used with susceptible, resistant and intermediate strains being available. The host factors govern the degree of resistance or susceptibility of mice to L. donovani (Bradley, 1974). Trotter et al. (1980) developed NMRI mouse model as a useful and easily manipulating laboratory model to examine potential antileishmanial (s), and established that these are among the most susceptible mice to infection with L. donovani. Similar results were observed by Bradley and Kirkley (1972)) and Bradley (1974). The commonly used strain, BALB/c mouse model was established by Reiner (1982), with highly reproducible levels of infection when an amastigote inoculum is administered i.v.. In 1984, Neal and Croft used the BALB/c mouse as a model for visceral leishmaniasis. Athymic and scid mice have been used as a model for treatment of VL in immunosuppressed cases (Marshall et al., 2000; Guerrero Figueroa et al., 2001). Other mouse models (CBA, C57/Bl) have been used (Samuelson et al., 1991) for lead optimization, that self-cure like most humans.

Mice infected with L. donovani have been widely studied, but this model does not reproduce the features of active human VL. In this animal, there is an early increase in parasite burden, but over the course of 4–8 week, the infected mouse is able to mount an antileishmanial cellular immune response and control the infection. This control is mediated by IFN-gamma (IFN-γ) production by splenic T cells (Murray, 1982; Murray et al., 1982; Squires et al., 1989), which are driven toward a Th1 phenotype by IL-12 (Murray, 1997). The generation of NO, through the up-regulation of inducible NO synthase (iNOS, NOS2) by IFN-g, is the critical macrophage effector mechanism involved in the control of parasite replication in the mouse (Green et al., 1990; Stenger et al., 1994). Thus, the murine model of L. donovani infection is a good model of early parasite replication followed by immunological control and subclinical infection, but there is no murine model for the progressive disease observed in human active VL.
1.7.2.2. The hamster model

Although many hamster species are susceptible to *L. donovani* infection (Smyly and Young 1924), the Syrian golden hamster (*Mesocricetus auratus*) establishes a good model for VL and provides a more synchronous infection in the liver and spleen that can develop into a chronic non-cure infection more similar to human VL (Farrell, 1976; Gifawesen and Farrell, 1989; Hommel et al., 1995). Gupta and Tiwari (2000) have reported the suitability and susceptibility of inbred hamsters in terms of parasite establishment and longer survival period as compared to outbred hamsters. The clinical evolution and immunological response in hamster to *Leishmania* infection has also been influenced significantly by gender related differences (Travi et al., 2002) these are probably due to the sex hormone status of the animal. Very recently, Dea-Ayuela et al. (2007) have studied the suitability and to establish suitable immunobiological parameters for in vivo testing of new antileishmanial compounds in the golden hamster model of visceral leishmaniasis.

The clinicopathological features of the hamster model of VL closely mimic active human disease. Systemic infection of the hamster with *L. donovani* results in a relentless increase in visceral parasite burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia, and ultimately death (Gifawesen and Farrell, 1989). Oliveira et al. (2004) demonstrated by their studies that the golden hamster is the best experimental model to study VL, because it reproduces the clinical and pathogenesis of the disease, as seen in humans and dogs. Unfortunately, the wide use of hamsters is still limited by the lack of available reagents such as antibodies to cell markers and cytokines. Al-Abdely et al. (1998) have cloned several hamster cytokine cDNAs to dissect the mechanisms related to progressive disease in this model, they reported that despite strong expression of the Th1-like cytokines in the liver, spleen, and bone marrow, there is uncontrolled parasite replication at these sites, leading to progressive disease. In the face of this strong IFN-γ expression, there was no detectable NOS2 mRNA expression or tissue enzyme activity, which is strikingly different from what was found in infected mice. Increased IL-4 expression was not observed in either the liver or spleen, but later in the course of infection, there was substantial production of the active form of TGF-β, IL-10, CD4+ and CD19+ cytokines that are known to suppress macrophage activation and generation of NO (Ding et al., 1990; Gazzinelli et al., 1992; Dea-Ayuela et al., 2007). Therefore, these observations indicate that progressive disease in this model is associated
with a defect in the generation of NO, an effector mechanism that is critical to the control of infection in the murine model.

1.7.2.3. The rat model

The cotton rat (*Sigmodon hispidus*) represents one of the most susceptible animal hosts for *L. donovani* (Fulton and Joyner, 1948). The infection remains 3-4 months and after the appearance of initial clinical signs, the disease progresses rapidly leading to death of the host. Mikhail and Mansour (1973), McKinney and Hendricks (1980) infected the African white tailed rat (*Mastomys albicanatus*) which proved to be an excellent host for *in vivo* maintenance and long term experiments with *L. donovani* and *L. braziliensis*. Nolan and Farrell (1987) have used *Mastomys natalensis*, a multimammatate rat as an experimental model for *L. donovani* and *L. major* and Dwivedi et al. (1983) successfully used this model for the infection of *L. donovani*. Kuwait rodents viz, *Mus musculus*, *Rattus norvegicus* have also been used as a model for leishmaniasis (Al-Taqi and Mohammed, 1981).

1.7.2.4. The dog model

Dogs have been used as an experimental model for *Leishmania* infections since the beginning of the century and experimental infections have also been achieved with *Leishmania* spp for which dog is not a natural reservoir e.g. *L. donovani* from India (Chapman et al., 1979). The infection of dogs with *L. infantum* or *L. chagasi* is an important laboratory model because it reproduces the natural infection similar to human infections (Rioux et al., 1969). German shepherd dogs are reported to give better results than beagles (Keenan et al., 1984), but some workers claim highly successful infection rate with mixed breeds (Abranches et al., 1991).

1.7.2.5. The primate model

Many primates have been experimentally used for infections with *Leishmania* spp viz. the owl monkey (Chapman et al., 1983), opossum (Hanson and Owsley, 1980) and the squirrel monkey (Chapman and Hanson, 1981; Dennis et al., 1985; Madindou et al., 1985; Dennis et al., 1986). Githure et al. (1986) worked on suitability of few east African primates for VL viz. vervet monkey, syben monkey and baboons. Anuradha et al. (1992) have successfully developed langurs (*Presbytis entellus*) as acceptable host of *L. donovani*.

A critical review of the literature would show that the golden hamsters take the *L. donovani* infection very well. These are sturdy animals and withstand repeated biopsies (Beveridge, 1963) thus they are suitable for studying the sequential effects of drug in the same animal. Additionally while working with *in vivo* models it was emphasized that,
organisms in the model should interact with the host immune system and with chemotherapeutic agents in a way comparable to that observed clinically. Golden hamsters and to a considerable extent BALB/c mice fulfill these requirements. The experimentation in these animals holds reasonable promise and if care is exercised, a good correlation could be obtained between the therapeutic efficacy in experimental hosts and clinical situation.

1.8. Antileishmanial screening techniques

Several screening techniques have been developed and adopted for antileishmanial drug testing. All have common procedure of assessing the efficacy against the parasite in different organs. However, the techniques differ in time interval between infection, drugging and therapeutic schedules. The well-recognized and documented techniques are briefly being described below.

1.8.1. Stauber's technique

Stauber et al. (1958) introduced the minimum time taking procedure of eight days for screening compounds against *L. donovani* in golden hamsters. It was for the first time intracardiac route of inoculation employed. In this technique, the target organ for assessment of activity was liver, because the parasites initially go to liver and then to deeper organs. The parasites are easily countable in liver smear as early as one hour post-inoculation. At this time, the initial parasitic burden may be ascertained on necropsy of few infected hamsters. The treatment commences on day 1 post-infection (p.i.) and continued for 6 days. Autopsy is done on day 8 p.i. (One day after the last dose of the treatment) and the parasite density in the liver is assayed. At this time, the parasitic burden in the untreated liver is 8 times higher than that of spleen. Efficacy of sample assayed was calculated, as total number of parasites in the organ (ratio multiplied by weight of organ in milligram) is plotted and total number of parasite is compared with the treated liver.

Although this technique allows quick assessment of antileishmanial compounds whilst, there are many lacunae. The drugs are administered before the infection is established, and as such it lays emphasis more on the extra cellular amastigotes, which is not the normal situation in established Kala-azar cases. It is not necessary that the drugs, which kill free amastigotes, will also affect amastigotes within the macrophages. Also the time factor is very short so, the activity of slow acting compounds could likely be missed. Since, the baseline parasite load is observed by sacrificing animals few hours after the inoculation there is always a margin of error due to variability in different animals.
1.8.2. Mikhail and Mansour’s technique (1975)

Mikhail and Mansour (1975) have followed the Stauber’s technique with slight modifications and initiated drug therapy on day 15 post- infection (p.i.). The animals were autopsied on day 60 p.i. and both liver and spleen were examined for parasites. A batch of untreated infected controls was killed on day 15 (on the day of drug initiation) to obtained baseline parasitic burden in spleen and liver. The other batch was sacrificed on day 60 p.i. for making comparison with treated animal.

This has certain advantage over the original technique of Stauber, as the treatment is initiated two weeks after the infection, giving sufficient time for establishment of infection and compound showing delayed activity can also be identified by this method.

1.8.3. Hanson’s technique (1977)

In Hanson’s technique (1977), which is a slight modification of Stauber’s method, the drug administration is initiated on day 3 p.i. instead of 24 hours, as in the case of Stauber’s method. Here too, considerable reduction in parasite load in the liver was observed but total cure was not achieved. The dose schedule was two doses a day for 6 days and the route of administration was intra-muscular (i.m.), intra-peritoneal (i.p.) or oral (p.o.). One day later the hamsters were killed, their livers were removed, weighed and dab smears were made. The antileishmanial activity of test compound was compared with that of the reference compound Glucantime. The Glucantime index (relative activity of the test compound to that of glucantime) for each test compound was calculated by the following formula-

\[
\text{Glucantime index (G.I.)} = \frac{\text{SD90 for Glucantime}}{\text{SD90 for test compound}}
\]

The only advantage with this technique is that it require lesser time to generate efficacy data and is therefore, economical but has similar disadvantages as discussed for Stauber’s method.

1.8.4. Beveridge’s technique (1963)

Beveridge (1963) introduced significant improvement to Stauber’s technique and suggested that potential compound should always be administered only after the infection has been established (3-4 weeks p.i.). A pretreatment biopsy was carried out for assessment of parasitic burden in spleen prior to therapy, allowing selection of experimental animals
with similar parasitic load. The animals were sacrificed on day 7 p.t., thus allowing sufficient time for drug action.

1.8.5. Technique of Raether et al. (1978)

In 1978, Raether et al. (1978) devised a technique, where the treatment was given for 6 days and the results obtained within a month. Here the parasites were observed in liver and the standard drugs showed moderate activity. Recently, Trotter et al. (1980) who used NMRI mice has shown good efficacy of pentostam and meglumine antimoniate.

1.8.6. Technique of Guru et al. (1978)

Although the Beveradge technique is most logical, it has a major disadvantage of missing out compounds showing delayed activity. Guru and co-workers modified the former technique with spleen biopsy on day 28 p.t. in addition to on day 7 and day 14 p.t. thus, facilitating assessment of the status of parasite at different intervals.

A critical appraisal of the screening techniques by Gupta et al. (1992) also shows that none of them is able to provide comprehensive information about the total efficacy of a potential drug. This is because the total effect of a drug is depending on two factors, (a) the effect of drug on the parasites, and (b) on host immune system. Many drugs are known to act through the immune machinery of the host.

In Stauber's and Hanson's techniques, the assessment is based on the effect of a potential drug on day 1 to 3 p.i. and that too on the parasite in liver only. This is far from actual situation in clinical practice where, the VL cases are more chronic and the parasites are located in deeper organs like spleen and bone marrow. Further, these methods completely ignore the host immune system.

Mikhail and Mansour (1975) started treating animals on day 15 p.i., the treated and control animals were sacrificed on day 60 post-infection. This technique can detect delayed action of a drug quite well.

Beveridge's (1963) method is more logical as the pre-treatment parasitic burden is assessed by spleen biopsy to select experimental animals carrying similar parasitic load. However, the animals are sacrificed on day 7 post-treatment (p.t.). It is, therefore, impossible to assess the delayed action of drugs. Guru et al. (1989) modified the technique where the delayed action of drugs can also be assessed conducting repeated spleen biopsies on the same animal at different intervals of day 7, 14, and 28. This is more rational as it
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gives all information regarding cure and survival time of treated animals and allowed sufficient time to the host immunity to play, if any, a role.

1.9. Immunomodulation

*Leishmania* infection is classically associated with down regulation of Th1 (Th-1) cells and preferential up regulation of Th helper type-2 (Th-2) cells. Thus, a shift of T helper cells towards Th-1 response would be a promising leishmanial therapeutic strategy (Awasthi et al., 2004). Cure of leishmaniasis, probably even during chemotherapy, appears to be dependent upon the development of an effective immune response. The macrophages produce toxic nitrogen and oxygen metabolites upon activation by interferon-gamma (IFN-γ) released by T helper cells kill the intracellular amastigotes (Murray et al., 1989; Alvar et al., 1997; Berhe et al., 1999; Joshi et al., 2002). IFN-γ is the first and only cytokine was tested in immunomodulation of VL so far. Studies showed that biological immunomodulators such as IFN-γ could provide a missing signal and enhance the activity of antimonials in the treatment of VL and CL. Recently, a new generation of immunopotentiating drugs have shown potential for leishmaniasis treatment. The imidazoquinoline imiquimod, an ingredient of the topical cream for genital warts known as Aladara™, induces nitric oxide (NO) production in macrophages was shown to has antileishmanial activity via macrophage activation in experimental models (Buates and Matlashewski, 1999) and in clinical studies on CL in combination with antimonials (Arevalo et al., 2001).

1.10. Natural products

Natural products are often overlooked in anti-protozoal chemotherapy. However, many antimicrobial antibiotics are important antiprotozoal drugs or have provided important leads in this area. In a recent review (Rocha et al., 2005) "101 plants, their families, and geographical distribution", the parts utilized, the type of extract and the organisms tested are discussed. It also includes 288 compounds isolated from higher plants and microorganisms, classified into appropriate chemical groups. Licochalcone A from the Chinese liquorice plant *Glycyrrhiza* has shown reasonable oral efficacy in experimental models of VL and CL; synthetic oxygenated derivatives are also active (Zhai et al., 1999). One derivative, 35 m4ac, resulted in 97% suppression of *L. donovani* liver amastigotes in a hamster model when given at 20 mg/kg for six days intraperitoneally. The compounds appear to interfere with mitochondrial function. The 2-substituted quinoline alkaloids, from the Bolivian plant
Galipea longiflora, have also shown oral activity in experimental VL and CL mouse models (Fournet et al., 1996). Saponins purified from the Vietnamese plant Maesa balansae and designated PX-6518 showed excellent activity after parenteral administration against VL and CL in rodent models (Guerin et al., 2002).

1.1.1. Combination therapy in VL

Growing resistance of the parasite to antileishmanial drugs suggest that the currently used monotherapy needs to be reviewed. Drug combinations have proven to be an essential feature of antimicrobial treatment through design or use to (i) increase activity through use of compounds with synergistic or additive activity, (ii) prevent the emergence of drug resistance, (iii) lower required doses, reducing chances of toxic side effects and cost, or (iv) increase the spectrum of activity, for example, the use of an antileishmanial with either an anti-inflammatory or immunodulator in cutaneous leishmaniasis (Croft et al., 2006). Multidrug combination treatment of VL, as practiced in tuberculosis and leprosy, should be given serious thoughts to prevent/delay the appearance of drug resistance. Previous studies on drug combinations for VL, for example, allopurinol plus sodium stibogluconate (Chunge et al., 1985) and paromomycin plus sodium stibogluconate (Chunge et al., 1990; Rudolph et al., 1995; Thakur et al., 2000), have aimed to improve efficacy. Although at present not many effective antileishmanial drugs are available, once oral miltefosine (which has been approved for the treatment of VL in India), sitamaquine and parenteral aminosidine become available, these drugs along with Amp B and Sb\textsuperscript{V} should be used in combination not only to combat drug resistance but also to shorten the duration of treatment. Thus, the northern districts of Bihar have the distinction of being unique in terms of large-scale Sb\textsuperscript{V} failure; resistance is likely to go up and spread into the areas where Sb\textsuperscript{V} is still effective. Rampant misuse of Sb\textsuperscript{V} (inadequate doses and insufficient duration) has led to the development of refractory strains, which tolerate several times more drug than those still responsive to it. More work is needed to identify the changes occurring in these strains and molecular tools for identification of these strains need to be developed. In areas with Sb\textsuperscript{V} resistance, it needs to be replaced with safer and more effective drugs. Unfortunately at the moment despite its limitations, amphotericin B is the only option for these patients. Oral miltefosine, which has been approved for the treatment of VL in India and should then be, used as first line-therapy (Sundar and Murray, 2005). When more clinically effective antileishmanial drugs are
developed, combination chemotherapy may hold the key to curing the emergence of drug resistance.

**1.12. Scope of the study**

Control of leishmaniasis is very difficult and challenging. Despite impressive advances in science, technology and medicine, we have until now not been successful in allocating sufficient resources to fight this dreadful disease that particularly affects the poor. Although drug management in leishmaniasis has evolved rapidly and with success, but obstacles continue to limit the impact of these advances in regions of endemicity (Murray, 2001). Lack of affordable new drugs, still a basic unsolved problem, has been joined by additional therapeutic obstacles including large scale resistance to pentavalent antimony (SbV) in India (Farault-Gambarelli et al., 1997; Lira et al., 1999) and co-infection with human immunodeficiency virus in all endemic regions. Available treatment options have actually expanded and includes successful application of less expensive generic SbV; rediscovery of the high level efficacy of amphotericin B; implementation of short course regimens of lipid formulations of amphotericin B; potential to replace SbV and amphotericin B with price capped paromomycin; and identification of the first effective oral agent, miltefosine. How to sustain and move this progress ahead remain difficult next steps in the treatment of leishmaniasis. The advances have been significant as the concept of choice for treatment is now real because, the currently available drugs are often toxic and very expensive. These shortcomings lead the researchers to put sincere efforts in the search of an alternative drug(s) to antimony. However, the development of new chemotherapeutic agent(s) is time consuming, expensive, cumbersome, and even today no accomplishment has been achieved towards the development of safer, reliable and affordable antileishmanial drug. The *in vitro* screening system provides rapid results to short out the lead from mass in comparison to *in vivo* method. Further, the *in vitro* screening system is less expensive, time saving and manageable in comparison to *in vivo* model and it is not logical to go for *in vivo* model. The current screening assays also have several shortcomings due to being either labour intensive or costly because of involvement of radio-labeled precursors or metabolic substrates (Kamau et al., 2001; Fumarola et al., 2004) and data are often hard to reproduce. Immunity of the host plays pivotal role in determining the therapeutic efficacy, since the immunity and drug effects are synergistic to each other (Chen et al., 1988; Murray et al., 1993). In leishmaniasis the host is severely immunosuppressed. Evidences are on record that once the infection is established the course of the disease in terms of chronicity is
determined by success and failure of immune system (Garnham and Humphrey, 1969). It has been demonstrated that an intact immune system is necessary for effective chemotherapy, which activates macrophages to produce toxic nitrogen and oxygen metabolites to kill the intracellular amastigotes (Murray et al., 1989; Alvar et al., 1997; Berhe et al., 1999). The effects of immunomodulators on both VL and cutaneous leishmaniasis have been studied either alone or in combination with chemotherapeutic drugs (Buates and Matlashewski, 1999; Arevalo et al., 2001). It has been reported that simple amino sugars have immunopotentiating activity (Khan et al., 2001) and glycosyl urea and many simple sugar derivatives possess property to modulate the enzymes involved in oxidation defense system of the parasite and do possess in vitro antiparasitic activity (Mishra et al., 2003).

Thus, an attempt has been made to establish the relationship between therapeutic responses and immune status of the host, employing diglycosylated ureides and the standard antileishmanial drug sodium stibogluconate (SSG).

Identification of novel drug targets is required for the development of new classes of drugs to overcome drug resistance and replace less efficacious treatments. The identification of genes involved in novel pathways or biochemical processes of the parasite that are clearly essential for life but fundamentally different from the pathways found in the host can provide an important lead to potential new drug targets. The metabolism is one of the most important core biological processes in cells by which small organic compounds (metabolites) sequentially interconverted by protein catalysts (enzymes). Among the metabolic pathways, glycolysis or energy metabolism is most interesting in research viewpoint, because glucose is the sole carbon source for trypanosomatids including *Leishmania* (Opperdoes and Borst, 1977). The enzymes of the glycolytic pathway are present in four human infecting species of *Leishmania* (Martin et al., 1976).

Among the enzymes of the glycolytic pathway hexokinase, the first enzyme, is of keen interest because it catalyses the transfer of the \( \gamma \)-phosphoryl group of ATP to a glucose molecule. Almost all glucose 6-phosphate so formed is metabolized by the glycolytic pathway, allowing for the synthesis of ATP, whereas remainder enters the pentose phosphate pathway (PPP) for the synthesis of NADPH and the precursors of nucleic acid. Thus, any inhibition of hexokinase would directly interfere with the formation of both ATP and nucleic acid precursors (Willson et al., 2002). The trypanosome enzyme differs from the host enzyme (36% - 37% identity with the three human hexokinase enzymes) in
that it is not inhibited by glucose-6-phosphate. These features render the hexokinase as interesting target for selective drug design. Also, hexokinase is a matrix protein sequestered inside the glycosome like other glycosomal enzymes with the help of a number of proteins called peroxins (PEXs) (Elgersma and Tabak, 1999). These peroxins are synthesized in the cytosol on free ribosomes recognize specific sequences in the matrix protein to be imported called peroxisome targeting signal (PTS) sequences. There are two well-defined signals; PTS1, the C-terminal tripeptide serine-lysine-leucine (SKL) or a variation thereof, and PTS2, a nona-peptide motif-R/K-L/V/I-X5-H/Q-A/L- close to the N-terminus, have been reported (Elgersma and Tabak, 1999). Nascent PTS1 and PTS2 polypeptides synthesized on cytosolic ribosomes are selectively bound by the mobile cytosolic receptors PEX5 or PEX7 respectively (Subramani, 1998; Purdue and Lazarow, 2001). These PEX5–PTS1 and PEX7–PTS2 complexes converge at the peroxisome-like microbody membrane where they dock to a receptor containing two core components, PEX13 and PEX14. The interactions between PEX5 and PEX14 of both T. brucei and L. donovani have been studied in detailed (de Walque et al., 1999; Jardim et al., 2000; Jardim et al., 2002; Choe et al., 2003; Moyersoen et al., 2003; Madrid et al., 2004; Madrid and Jardim, 2005). In trpanosomatids, HK is a PTS2 targeting protein which requires PEX7 for its targeting into glycosome and the amino-acid motifs of the targeting signals are rather well conserved. PTS-2 sequences form two closely intertwined structures with the receptor. Any inhibitor binding to this specific structure would interfere with the PTS-2-Pex7 interaction and thus prevent matrix protein from entering glycosomes (Galland et al., 2007). Thus inhibitors designed can selectively interfere with these interactions are expected to prevent the synthesis of functional glycosomes and therefore kill the parasites.

Therefore, there is an urgent need for the development of more selective and efficacious drug and identification of potential drug target for antileishmanial therapy that are unique to the parasite. For these purposes the following objectives are-

1. Development of new in vitro screening models using Leishmania donovani cell line expressing luciferase reporter gene
   (a) Evaluation of standard antileishmanials and CDRI compounds for their antileishmanial activity against Luc- transfected promastigotes.
   (b) Standardization of quantitative, reliable and rapid semi in vivo screening system using Luc-macrophage amastigote system and evaluation of standard antileishmanials and CDRI compounds.
2. Impact of adjunct therapy of immunomodulators with known antileishmanial using *in vitro* (macrophage-amastigote) and *in vivo* model of *L. donovani*

   (A) Assessment of therapeutic/adjunct efficacy in *L. donovani* hamster model.

   (B) Monitoring of macrophage functions and biochemical parameters before, during and after treatment.