Antileishmanial treatment using chemotherapy in combination with immunomodulators in experimental Visceral Leishmaniasis

THESIS

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CONTENTS

ABBREVIATIONS

%	**Percent**	**PECs**	**Peritoneal exudate cells**
°C	**Degree centigrade**	**EDTA**	**Ethylene diamine tetra acetic acid**
h	**Hours**	**BSA**	**Bovine serum albumin**
min	**Minutes**	**RPMI**	**Roswell Park Memorial Institute**
sec	**Seconds**	**MEM**	**Minimum essential media**
kb	**Kilo bases**	**FBS**	**Fetal bovine serum**
kDa	**Kilo Dalton**	**HEPES**	**N-[2-hydroxyethyl] pipeazine-N'-[2-ethanesulfonic acid]**
lbs	**Pounds (pressure)**	**FITC**	**Fluorescein isothio cyanate**
Mg	**Milli grams**	**PI**	**Propidium Iodide**
µg	**Micro gram**	**DCFH**	**Dichlorofluorescein diacetate**
ml	**Milli liters**	**DAF2DA**	**Diaminofluorescein-2-diacetate**
µl	**Micro liter**	**PMA**	**4á-Phorbol 12-myristate 13-acetate**
µM	**Micro Molar**	**Con A**	**Concanavalin A**
nM	**Nano Molar**	**OD**	**Optical density**
mM	**Milli Molar**	**H_2O_2**	**Hydrogen peroxide**
mCi	**Milli Curie**	**NO**	**Nitric Oxide**
cm	**Centimeter**	**ROS**	**Reactive oxygen species**
m	**Meter**	**RNS**	**Reactive nitrogen species**
rpm	**Revolutions per minute**	**L-NAME**	**N-nitro-L-arginine methyl ester**
cpm	**Counts per minute**	**PTx**	**Pentoxifylline**
pH	**Negative log of hydrogen ion**	**PI**	**Percent Inhibition**
MW	**Molecular weight**	**SI**	**Stimulation index**
TDW	**Triple distilled water**	**IP**	**Intraperitoneal**
PBS	**Phosphate buffered saline**	**PO**	***Per os***
DMSO	**Dimethyl sulfoxide**	**IC**	**Intracardiac**

PREAMBLE

Leishmaniases, in its variety of visceral (VL), cutaneous (CL) and mucocutaneous (MCL) forms, directly affects about 2 million people per annum, with approximately 350 million individuals at risk worldwide. During the last 10 years there have been extensive epidemics of visceral form of the disease, which is also emerging as an important opportunistic infection in immunocompromised patients, especially those co-infected with HIV. No vaccines exist for VL, CL or MCL and chemotherapy is inadequate and expensive. In the present scenario 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, (iv) increase the spectrum of activity. The work of the present Ph.D. thesis has been divided in to six chapters. Each chapter deals with a specific objective. The 1st chapter provides general information regarding Leishmaniasis and its parasite, history, taxonomy, global surveillance, epidemiology, life cycle, vector and transmission. The 2nd chapter deals with an exploratory review on conventional therapy for VL, molecular mechanism involve in parasite invasion and host immune response during leishmaniasis. Chapter 3rd gives detail information regarding the application of combination therapy for treatment of the disease. The 4th chapter provides detail description of materials and methods used during study. The 5th chapter describes combination therapy using immunomodulator CpG oligodeoxynucleotides with miltefosine in *L.donovani*/hamster and BALB/c mouse model. The 6th chapter explains combination therapy using immunomodulator and hepatoprotective agent picroliv with paromomycin and miltefosine in *L.donovani*/hamster model.

CHAPTER-1

LEISHMANIASIS: AN OVERVIEW

1.1. Introduction

Leishmaniases refer to a constellation of vector-borne disease, which is caused by obligate intracellular protozoan parasites of the genus Leishmania. This disease is a severe public health problem in tropical and subtropical regions of the world. Major characteristic of this disease is its diversity and complexity (Herwaldt, 1999). More than 20 species of Leishmania cause leishmaniasis and it is transmitted to humans by ~30 different species of phlebotomine sandflies (Pearson *et al.,* 1996). Leishmaniasis is classified as one of the ‘‘most neglected diseases’’ (Yamey & Torreele, 2002) based on the limited resources invested in diagnosis, treatment, and control, and its strong association with poverty (Alvar *et al.,* 2006a). The disease is second in mortality and fourth in morbidity among all tropical diseases (Bern *et al.,* 2008).

Leishmaniases has several diverse clinical manifestations: Cutaneous Leishmaniasis (CL) - ulcerative skin lesions, Mucocutaneous Leishmaniasis (MCL) - destructive mucosal inflammation and Visceral Leishmaniasis (VL) - disseminated visceral infection, each presenting distinct diagnostic challenges, most requiring prolonged, expensive drug therapy and each contributing differently to disease burden. Post kala azar dermal leishmaniasis (PKDL) is characterized by macular, maculo papular or nodular rash and is a complication of VL that is frequently observed after treatment. Interactions with malnutrition and HIV alter the clinical course, and complicate therapeutic strategies. In the absence of highly active antiretroviral therapy (HAART), the relapse rate after treatment approaches 100%. Other complicated forms include disseminated cutaneous leishmaniasis (DCL) recognized by diffuse nodular non-ulcerating disease, and leishmaniasis recidivans (LB) characterized by localized and slowly progressive non-healing lesions. Both are rare, difficult to treat, and can be severe. Among these VL is the most severe. It is caused by *L. donovani* in the Indian subcontinent, Asia, and Africa and by *L. infantum* or *L. chagasi* in the Mediterranean region,

southwest and central Asia, and South America (Murray, 2005). It is characterized by progressive fever, weight loss, splenomegaly, hepatomegaly, hypergammaglobulinemea and pancytopenia. Complications include immunosuppression and secondary bacterial infections, hemorrhage, anemia and during pregnancy, it causes foetal wastage or congenital leishmaniasis (Pagliano *et al.,* 2005). In short Kala-azar is 100% fatal if, left untreated (Desjeux, 1996). Even in treated patients, case-fatality rates are often 10% or higher; jaundice, wasting, severe anemia, and HIV co-infection are associated with increased risk of mortality (Collin *et al.,* 2004; Bern *et al.,* 2005; Rey *et al.,* 2005).

The recommended drugs for VL & CL were the antimonials, first introduced 75 years ago (Deps *et al.,* 2000) however, lack of response to pentavalent antimonials actively wide spread in India and Sudan led to the use of Amphotericin -B or Pentamidine. It is unlikely that one single drug or drug formulation will be effective against all forms of leishmaniasis since (a) the visceral and cutaneous sites of infections impose varying pharmacokinetic requirements on the drugs to be used and (b) there is an intrinsic variation in drug sensitivity of the 20 Leishmania species known to infect humans. In addition, there are other new problems to be surmounted by novel treatments, namely: (i) the need for drugs for treatment of VL in Bihar State, India, where there is acquired resistance to the pentavalent antimonials and (ii) the need for treatment for VL and CL in immunocompromised paitents, in particular due to HIV co - infection, where there is exacerbation of disease or emergence from latent infection due to the depleted immune response. In the latter case standard chemotherapy is frequently unsuccessful (Alvar *et al.,* 2006b). Among the new drugs discovered miltefosine and paromomycin are at top priorities. Miltefosine, a hexadecylphosphocholine, is the first promising oral drug which can be used against leishmaniasis and a major milestone in chemotherapy of VL. It was initially developed as an anticancer agent, quickly and effectively eliminated Leishmania promastigotes from culture. Attention to this compound

led to preclinical and clinical studies conducted for leishmaniasis (Croft *et al.,* 1987). As a result, miltefosine has been registered for the treatment of VL in Germany and India in 2004, as well as for CL and VL in Colombia. Miltefosine, is although an effective oral drug but its use in women of child-bearing age is restricted due to teratogenicity. In addition, it has a long half-life, which might encourage the emergence of resistance once its use becomes widespread (Bryceson, 2001). Second strong candidate, Paromomycin (formerly known as aminosidine) is an aminoglycoside active against Gram-negative and many Gram-positive bacteria as well as some protozoa and cestodes. The antileishmanial properties of paromomycin were recognized by Kellina in 1961 (Kellina, 1961) and were confirmed by Neal *et al.,* (1968 & 1995). It was registered in India in August, 2007 for treatment of VL (den Boer and Davidson, 2006). The results from initial studies in India were promising (Chunge *et al.,* 1990; Jha *et al.,* 1998). Currently, the non-profit group Drugs for Neglected Diseases Initiative is conducting studies on paromomycin (as monotherapy and in combination) in VL in Africa, and the Institute for One World Health is conducting a Phase IV study in India (Davidson *et al.*,2008). Other drugs such as sitamaquine, azoles and azythromycin have been reported as having variable cure rates. Consequently, there is still a real need for new active compounds that can provide therapeutic benefits but with fewer side effects (Pape, 2008).

1.2. History of Visceral Leishmaniasis

Descriptions of conspicuous lesions similar to CL have been discovered on tablets from King Ashurbanipal from the 7th century BC, some of which may have been derived from even earlier texts from 1500 to 2500 BC. Muslim physicians including Avicenna in the 10th century gave detailed descriptions of what was called Balkh sore (Cox, 1996). In 1756, Alexander Russell, after examining a Turkish patient, gave one of the most detailed clinical

descriptions of the disease. As for the new world, evidence of the cutaneous form of the disease was found in Ecuador and Peru in pre-Inca potteries depicting skin lesions and deformed faces dating back to the first century AD. 15th and 16th century texts from the Inca period and from spanish colonials mention *"valley sickness"*, *"Andean sickness"* or *"white leprosy"* which are likely to be CL (WHO, 2007). Physicians in the Indian subcontinent would describe it as Kala-azar. Kala-azar first came to the attention of Western doctors in 1824 in Jessore, India, where it was initially thought to be a form of malaria. India gave kala-azar its common name, which is the Hindi for "black fever", so called for the darkening of the skin on the extremities and abdomen that is a symptom of the Indian form of the disease. The agent of the disease was also first isolated in India by Scottish doctor William Leishman and Irish physician Charles Donovan, working independently. As they published their discovery almost simultaneously, the species was named for both of them - *Leishmania donovani*. Today, the name kala-azar is used interchangeably with the scientific name visceral leishmaniasis for the most acute form of the disease caused by *L. donovani*. Along with India this disease is seen at its most deadly in north and east Africa. It can also be found throughout the Arab world and southern Europe. But, while the disease's geographical range is broad, it is not continuous. The disease clusters around areas of drought, famine, and high population density. In Africa common infection centers are in Sudan, Kenya, and Somalia (Francois, 1995).

1.3. Taxonomy

The classification of Leishmania was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties and clinical manifestations (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).

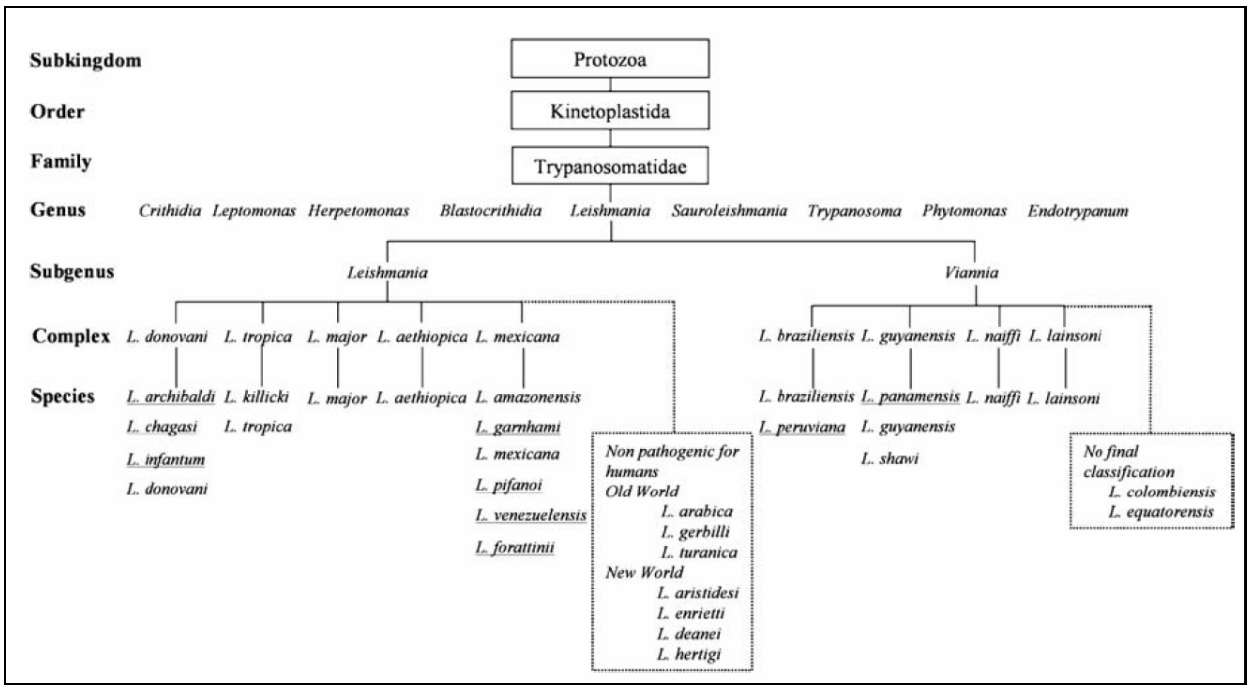


Fig.1. Taxonomy of Leishmania

Source: Based on the scheme published by the WHO, 1990

A modern scheme of classification of Leishmania is shown in Fig.1. All members of the genus Leishmania 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 iso-enzyme 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 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. archibaldi, L. garnhami, L. pifanoi, 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.

1.4. 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). Near about 90% of VL cases occur in India, Bangladesh, Nepal, Sudan, Ethiopia, and Brazil, while 90% of CL occurs in Afghanistan, Algeria, Iran, Saudi Arabia, Syria, Brazil, Colombia, Peru, and Bolivia (Desjeux, 2004; Modabber *et al.*, 2007). The distribution is dynamic: Colombia and Ethiopia have recently joined this list, and Pakistan currently faces a large epidemic of CL in Baluchistan and Sindh (Bern *et al.*, 2008). Climate change and other environmental changes have the potential to expand the geographic range of the vectors and leishmaniasis transmission in the future (Patz *et al.*, 2000).

1.4.1. Current situation in India

In the Indian subcontinent (Bangladesh, Nepal and India), the most common endemic form of the disease is 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 Jessor district of East Bengal (now in Bangladesh) (Sen Gupta, 1944; Peters and Prasad, 1983).

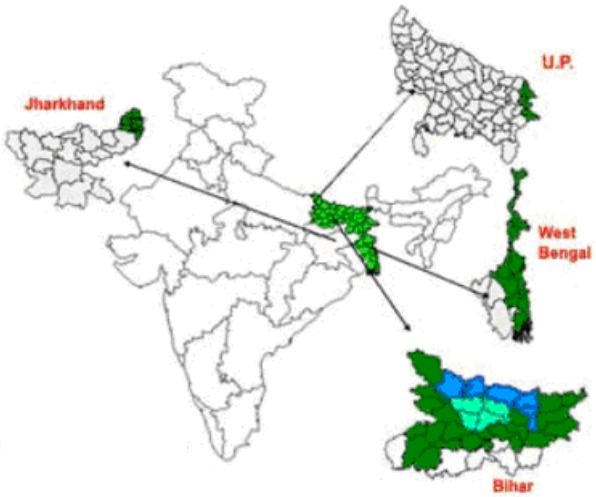


Fig.2. Areas effected with VL in India.

Source: www.indg.gov.in

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) and in 1989, 30,000 cases with 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 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 and10 districts of West Bengal, 40 out of total 54 districts in Bihar are badly affected with VL (Fig.2). 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. 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 V L 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; Shivaramakrishnamaiah 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 ºC and above 4 ºC with diurnal variation less than 10 ºC

e) Abundant vegetation with subsoil water

f) Rural setting.

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

1.5. Disease and its types

Leishmaniases 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). 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 leishmaniases can be divided into following general clinical patterns.

1.5.1. Cutaneous Leishmaniasis (CL)

CL is commonly known as oriental sore. Its causative agents are *L.major, L. tropica, L. aethiopica* 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 (Fig.3). It is frequently self-healing but when the

lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma. After recovery or successful treatment, cutaneous leishmaniasis induces immunity to re-infection by the species of Leishmania that cause the disease. It is prevalent in Mediterranean Basin, Syria, Arabia, and Mesopotamia, Persia to Central Asia, Central Africa and some parts of Western India. There are two chronic forms.

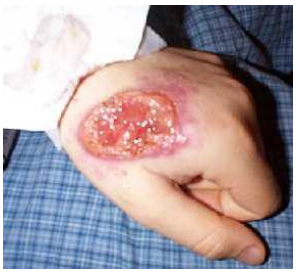


Fig.3. Leisons in cutaneous leishmaniasis

Source: www.pudsandlosers.blogspot.com

1.5.1.1. Diffuse cutaneous Leishmaniasis (DCL)

It is difficult to treat DCL due to disseminated lesions that resemble leprosy and do not heal spontaneously (Fig.4). This form is especially related to a defective immune system and it is often characterized by relapses after treatment.It is rare and disfiguring. Widespread plaques containing huge numbers of amastigotes persist for decades. It is caused mainly by *L. aethiopica* in Africa and *L. amazonensis* in South and Central America.

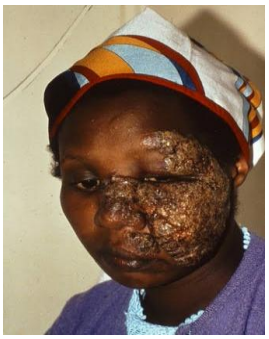


Fig.4 Facial lesion in diffuse cutaneous leishmaniasis

Source: http://pathmicro.med.sc.edu/parasitology/blood-proto

1.5.1.2. Leishmaniasis recidivans

It is a chronic, non-healing or relapsing cutaneous infection caused mainly by *L. tropica* in the Middle East. Its most severe form, recidivans leishmaniasis, is very difficult to treat, long lasting, destructive and disfiguring (Fig.5).

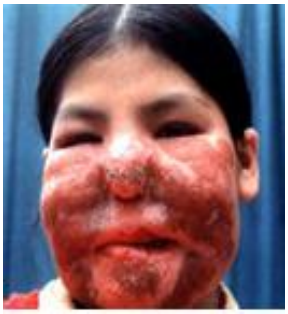


Fig.5 Facial lesion in leishmaniasis recidivans.

Source: http://pathmicro.med.sc.edu/parasitology/blood-proto

1.5.2. 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 (Fig.6).

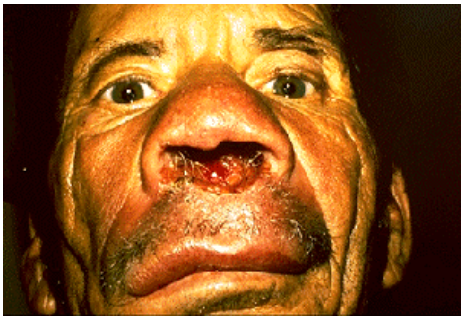


Fig. 6 Deformity in nasal mucosa due to mucocutaneous leishmaniasis

Source: www.stanford.edu.com

The soft tissues and cartilage of the oronasal/pharyngeal cavity undergo progressive erosion. Contrast to cutaneous leishmaniasis, these lesions does 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.

1.5.3. 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*. Infected 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, lymphoadenopathy, occasional acute abdominal pain, emaciation, anemia, leucopenia, and blackness of skin, hence the name given kala-azar or black-fever (Fig.7).

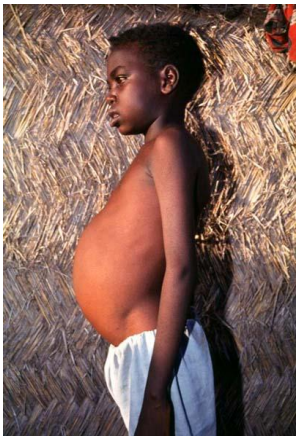


Fig. 7 Enlargement of Liver and Spleen in visceral leishmaniasis

Source: www.pathmicro.med.sc.edu

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, diarrhoea 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.

1.5.4. 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 (Fig.8), developed in about 5-15 % of kala-azar patients generally one or two years after completion of antimonial treatment (Rees and Kagar,

1987; Salotra *et al.,* 2006). It is characterized by development of macules, papules or nodules on the skin. PKDL in India resembles lepromatous leprosy with verrucous papilomatous, xanthomathous 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).

Fig.8 Nodular lesions on face and other body parts in PKDL

Source: www.icp.ucl.ac.be.com

1.6. Epidemiology and Ecology

Transmission of leishmaniasis to humans occurs through sylvatic, domestic, and peridomestic cycles. Sylvatic cycles involve an animal host which act as indefinite reservoir for the disease and maintain enzootic transmission without human disease. Such hosts are common in New World rain forests and the deserts of Central Asia. Disease gets transmitted to humans only when they enter the sylvatic habitat for economic or military purposes or when human habitation encroaches on the sylvatic setting. In domestic cycles, humans or dogs form the predominant or sole infection reservoir. Female sand fly of genus Phlebotomus in the Old World and Lutzomyia in the New World are the only proven vector responsible for transmission of the disease (Berman, 1997). In India, *Phlebotomus argentipes* is the only proven vector for the disease. Female sandflies (*Phlebotomus* and *Lutzomyia* spp.) get infected by taking a blood meal from infected human beings (anthroponoses) or terrestrial mammals (zoonoses). Imbibed amastigotes transform in the sandfly gut and replicate as

promastigotes; at a subsequent bloodmeal, metacyclic promastigotes are regurgitated and injected into the skin to complete the cycle (Rogers and Titus, 2004). About 70 of around 1000 known sandfly species transmit leishmaniasis. Vector competence in most species seems to be controlled by parasite ability to resist proteolytic enzymes during bloodmeal digestion and avoid excretion by binding to midgut epithelium. Binding is mediated by promastigote surface lipophosphoglycan and the phosphoglycan domains differ between species (Sacks, 2001). Sandfly saliva affects local host immune responses, promoting experimental cutaneous infection (Sacks and Noben-Trauth, 2002). 500,000 new cases for VL and more than 50,000 deaths from the disease every year have been reported (Desjeux, 2004). Such a death toll 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.,* 2006a). The majority (>90%) of cases occur 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 approx 100,000 people out of a population of 280,000 between 1984 to 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. Leishmaniasis cause considerable morbidity and mortality. It is a typical example of an anthropozoonosis. The majority of infections are originally zoonotic, although some cases are known for transmission of *L. donovani* from human to human. In a primitive or sylvatic cycle human infection is accidental, transmission occurring in wild foci, e.g. *L. braziliensis*; in 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 in a tertiary, strictly anthroponotic cycle the animal reservoir disappear and disease transmit from infected human to healthy human by sandflies, e.g. *L. donovani*. The epidemiology of leishmaniasis in a given area is directly dependent on the behaviour of the human and/or animal population in relation to the cycle of transmission. The foci that account for the largest number of human cases, for example, VL in South Asia and CL in Afghanistan, usually reflect anthroponotic transmission (Reithinger *et al.,* 2003; Jeronimo *et al.,* 2006). In anthroponotic VL foci, the reservoir includes humans with untreated kala-azar (Bern *et al.,* 2005) but PKDL patients may maintain the infection between kala-azar epidemics (Addy and Nandy, 1992). Up to half the population in highly affected foci may have asymptomatic leishmanial infection; the contribution of such individuals to transmission is presumed to be less than for active kala-azar, but has never been quantified (Costa *et al.,* 2002; Bern *et al.,* 2007). 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).

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.

• 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.

1.7. Vector (Sandfly)

Fig.9 Sandfly transferring parasite during blood meal

Source: www.albaeco.com

1.7.1. 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 sand fly transmits the protozoa, infecting it 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 (Fig.9). 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*. 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. perfiliew*i in Greece, *P. orietalis* 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.

1.7.2. Distribution of vector in India

Fauna of Indian sub-zone is represented by 46 species out of these 11 species belong to *Phlebotomine* species and 35 to *Sergentomyia* species. *Phlebotomus argentipes* is the proved vector of kala-azar (visceral leishmaniasis, VL) in India (Bhattacharya *et al.,* 2006).It prefers to hot and humid climates in all the VL abundant endemic areas of Bihar, West Bengal, Assam and Eastern Uttar-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 (Uttaranchal) and 1100 m in Nilgiri Hills (Tamil Nadu).

1.7.3. 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 and cattle shed (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 2.7m from the ground. It is found throughout the World inter tropical and temperate regions.

1.7.4. 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.

1.7.5. Vector control strategies

Control of VL mainly depends on its epidemiological features. In India, Bangladesh and Nepal where visceral leishmaniasis is anthroponotic, vector is controlled by chemical and environmental control measures.

1.7.5.1. Environmental control

Sandflies breed in dark corners in the crevices of the walls having rich humus and moisture. The principle behind the environmental control is to manage the environment to make it unsuitable for breeding. Vyokov in USSR successfully controlled leishmaniasis by destroying rodent burrows (Vyokov, 1980). In a study on technological control of sandflies, the walls of the resting sites were plastered filling all the cracks and crevices by mud and lime, and the breeding of sandflies could be stopped successfully (Kumar *et al.*, 1995). Lime has a powerful water absorbing capacity which makes it unsuitable for the sandfly breeding. In another experiment 70% population of *P. papatasi* successfully controlled by constructing cement skirting of 9" vertically on the wall and 9" horizontally on the floor (Dhiman, 1995).

1.7.5.2. Chemical Control

Under National Vector Borne Disease Control Programmes, DDT was extensively used for Indoor residual spraying, which is a simple and cost-effective method of controlling vector. DDT remains the insecticide of choice because of its low cost, high efficacy, long residual action and relative safety when used for indoor residual spray. Dosage schedule of 1 or 2 g/m^2 or 100-200 mg/ft^2 has been found to be quite effective; 5 per cent emulsified suspension

of DDT is the choice. 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).

1.7.5.3. Biological control

Very scanty information is available on the biological control of sandfly. As the application of biolarvicides in the field condition is difficult due to diverse breeding habitat of sandfly, their practical application appears to be of limited use in the control of visceral leishmaniasis. In the laboratory experiment De Barjac *et al.* (1981) first time demonstrated the role of *Bacillus thuringiensis* var. israelensis in the control of larvae of *P. papatasi* and *Lutzomyia longipalpis*. Robert *et al.*, (1998) successfully used *Bacillus sphaericus* in the control of *P. martini* in Kenya based on the work of Schlein (1987) and Yuval & Warburg (1989). This approach requires further evaluation.

1.7.5.4. Remote sensing

Satellite remote sensing has been successfully used in the identification of high risk areas. A pilot study has been concluded at RMRIMS, Patna, for identifying and mapping of *P. argentipes* distribution for early prediction of disease with the help of satellite remote sensing in integration with geographical information system (GIS) (Palit *et al.*,2001).

1.7.5.5. Use of insecticide impregnated bed nets in the control of leishmaniasis

Insecticide impregnated bed nets (ITN) were shown as one of the most effective methods of reducing man-vector contact and intra and peridomicilliary transmission of vector-borne diseases. In most studies the insecticides used were synthetic pyrethroids (permethrin, deltamethrin, Lambda-cyhalothrin), which combine the properties of low to moderate mammalian toxicity, low volatility and high insecticidal activity (WHO tech report, 1990). ITNs act as ‘baited traps’ but have also important deterrent and repellent effect. Compared with house spraying ITNs have theoretically the following advantages: (*i*) their effectiveness

is independent of the endophilic and exophilic behaviour of the vectors; (*ii*) less insecticide is used; and (*iii*) the household exerts control over its application and thus depends less on the performance of a knock-down planned disease control programme. 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). The principle of an ITN combines the effect of individual protection and insect-killing activity while a strong repellent effect could possibly enlarge its efficacy by reducing indoor and peri-domestic vector density. ITNs therefore have the potential to achieve individual protection for VL and users are not dependent on a top-down, government led intervention. The new long-lasting impregnated bed nets make yearly re-impregnation no longer necessary (Ostyn *et al.,* 2008)

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).

1.7.5.6. 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).

1.8. Transmission

1.8.1. Life cycle

During their complex life cycle, the single cell 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, promastigotes and amastigotes, which are found in vertebrate hosts and invertebrate hosts, respectively (Fig.10).

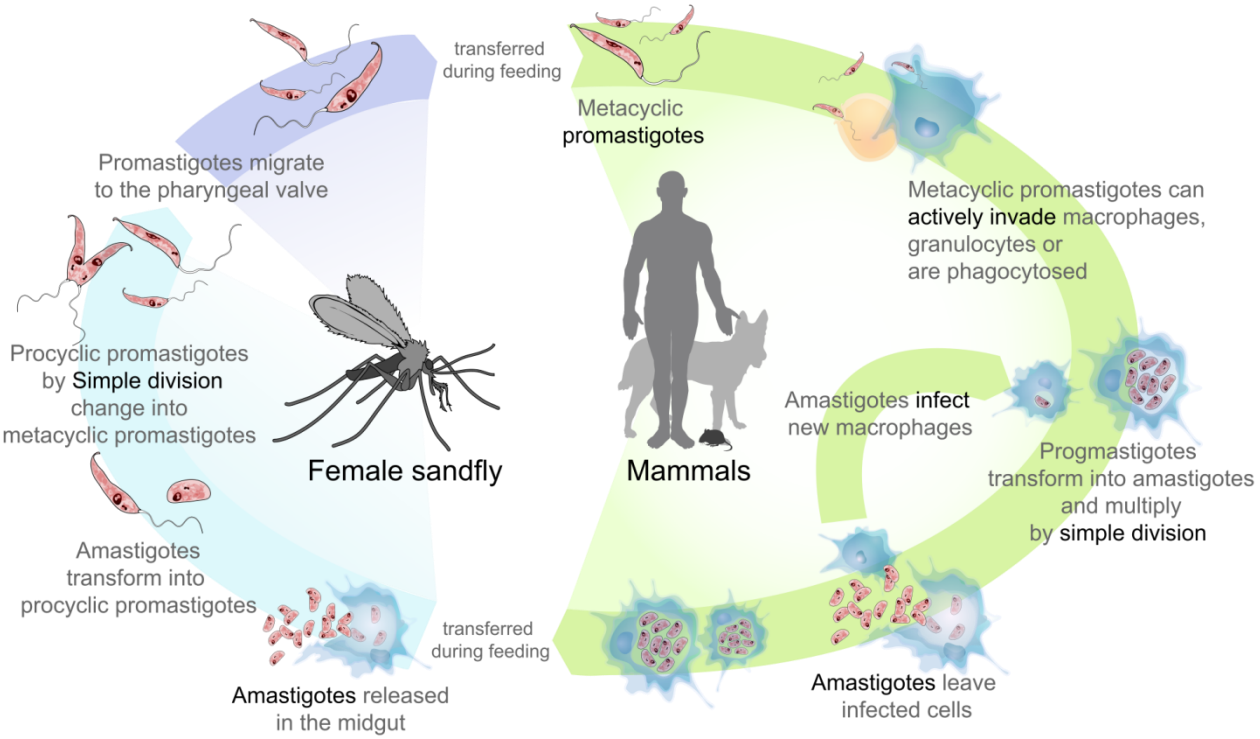


Fig.10 Life cycle of Leishmaniasis

Source: www.commons.wikimedia.org

1.8.2. Stages in the Invertebrate Host (Promastigote)

Like female mosquitoes, the female sandfly needs a blood meal for egg development and it 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 1-4µm, elongated motile extracellular stages possessing a prominent free flagellum up to 20 µm long. Nevertheless, a variety of different promastigote forms have been distinguished on morphological grounds (Bates and Rogers, 2004).

1.8.3. Stages in the Vertebrate Host (Amastigote)

In the vertebrate host, the parasite evolves into an amastigote form. Amastigotes are ovoid (2.5–5 µm 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 sand fly, due to change in temperature and other conditions, develop in to flagellated promastigotes and the life cycle continues.

1.8.4. Survival strategy of parasite

1.8.4.1. In Sandfly

Female sand flies acquire Leishmania parasites when they feed on an infected mammalian host in search of a blood meal. Sand flies are pool feeders, meaning they insert their saw-like mouthparts into the skin, and agitate them to produce a small wound into which the blood flows from superficial capillaries (Lane, 1993). It is this tissue damage associated with the creation of the wound that releases skin macrophages and/or freed amastigotes into the pool of blood, and enables their subsequent uptake into the abdomen of the sand fly. The change in conditions moving from the mammalian host to the sand fly midgut (decrease in temperature, increase in pH) triggers development of the parasite in the vector (Bates and Rogers, 2004; Kamhawi, 2006). The amastigotes transform into motile promastigotes with flagella beating at the anterior end (Fig.11a). This first stage in the vector is called a procyclic promastigote – it is a weakly motile, replicative form that multiplies in the blood meal (Fig.11b). This initial ‘‘blood meal phase’’ is confined by the peritrophic matrix, a chitin and protein mesh secreted by the midgut epithelium that encloses the blood being digested within (Secundino *et al.,* 2005). After a few days, the parasites begin to slow their replication and differentiate into elongate, strongly motile nectomonad promastigotes. These are migratory forms that accumulate at the anterior end of the peritrophic matrix and break out of the bloodmeal. This escape is facilitated by the action of a parasite secretory chitinase (Schlein *et al.,* 1991; Shakarian and Dwyer, 2000) and probably by the action of endogenous sand fly chitinase (Ramalho-Ortigao *et al.,* 2005). They move towards the anterior midgut, some of them attaching to the microvilli of the midgut epithelium, until they reach the stomodeal valve (cardia) that guards the junction between foregut and midgut. These nectomonad

promastigotes mediate the establishment phase of the infection that marks a true vector i.e. persistence beyond the blood meal and avoidance of expulsion during defecation. Thus the ability to attach is an important property of Leishmania promastigotes (Sacks and Kamhawi, 2001). Glycoconjugate lipophosphoglycan (LPG) present on parasite surface is responsible for binding to a galectin on the sand fly gut epithelium in certain species e.g. *L. major* in *Phlebotomus papatasi* (Pimenta *et al.*, 1992; Kamhawi *et al.*, 2004). Once they reach the stomodeal valve the nectomonad promastigotes transform into leptomonad promastigotes, shorter forms that resume replication (Gossage *et al.*, 2003). These are responsible for the secretion of promastigote secretory gel (PSG) (Rogers *et al.*, 2002), which plays a key role in transmission. Some of the nectomonad/leptomonad promastigotes also attach themselves to the cuticle-lined surface of the valve and differentiate into haptomonad promastigotes (Killick-Kendrick *et al.*, 1999). This form of attachment is mechanistically different to that seen with the midgut epithelium and is mediated by expansion of the flagellar tip into hemi-desmosome-like structures (Vickerman and Tetley, 1990; Wakid and Bates, 2004). Finally, some of the leptomonads differentiate into metacyclic promastigotes (Rogers *et al.*, 2002), which are the mammal-infective stages. These are deposited in the skin of a new mammalian host when the fly takes another blood meal, leading to the transmission of disease.

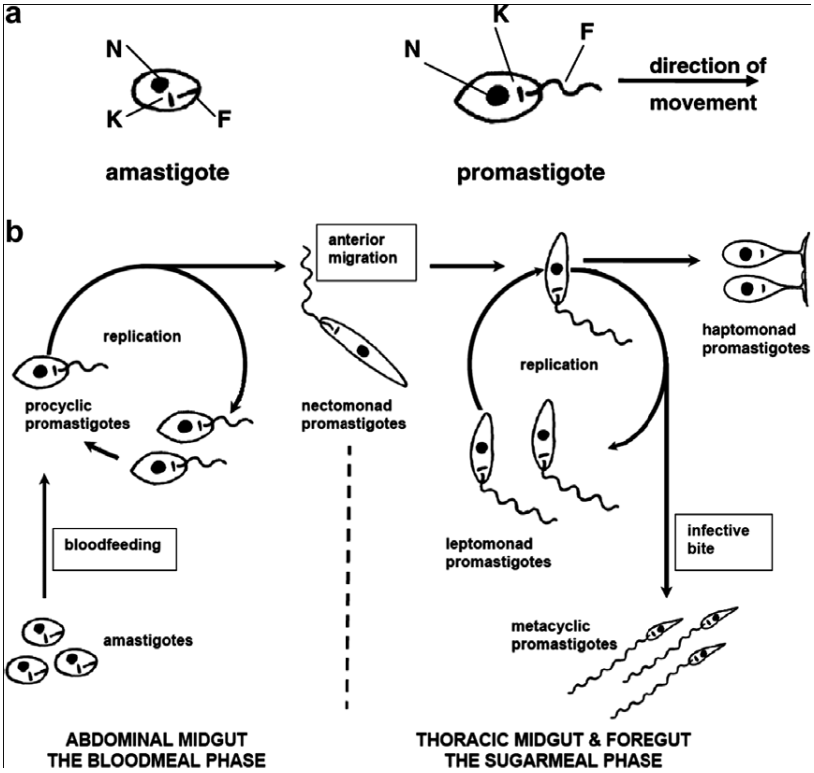


Fig.11 Development of Leishmania parasite in the sand fly vector (Bates, 2007)

(a) The morphology of amastigotes and promastigotes. Each form has a nucleus (N), kinetoplast (K) and flagellum (F). The kinetoplast is the mitochondrial genome. The flagellum in amastigotes is internal and non-functional; in promastigotes the flagellum extends from the cell body, beats and pulls the organism in the direction shown, emerging from the anterior end of the cell.

(b) The developmental sequence of the five major promastigote forms: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes and metacyclic promastigotes. The exact position of haptomonad promastigotes in the developmental sequence is uncertain.

1.8.4.2. In macrophages

Survival of intracellular infectious pathogens in the host organism is best achieved when the pathogen resides in a cell type that *per se* is not able to exert antimicrobial activities. In Leishmania infections a number of cell types were postulated to function as 'safe targets' during long-term persistence. These include immature myeloid precursor cells and monocytes (Mirkovich *et al.,* 1986), sialoadhesin-positive stromal macrophages of the bone marrow (Leclercq *et al.,* 1996; Cotterell *et al.,* 2000), hepatocytes (Gangneux *et al.,* 2005) and fibroblasts (Bogdan *et al.,* 2000b). Promastigotes are phagocytosed by macrophages, either directly or after infection of neutrophils initially recruited to the sandfly bite (van Zandbergen

et al., 2004). Promastigotes are targeted to vacuolar compartments in the macrophage that have the characteristics of mature phagolysosomes, where they differentiate to the smaller aflagellated amastigote stage. Amastigotes proliferate by binary cell division and can spread to other macrophages as well as some other phagocytic (i.e. dendritic cells) and no-professional phagocytic (i.e. fibroblasts) cells. The capacity of these pathogens to target and replicate within the mature phagolysosome compartment is remarkable. LPG is the primary ligand for multiple macrophage opsonic and pattern recognition receptors (Naderer *et al.,* 2004). LPG plays a critical role in macrophage infection it protects promastigotes from a transient rise in reactive oxygen species (ROS) generated during phagocytosis (Spath *et al.,* 2003). LPG may scavenge ROS directly and/or inhibit macrophage signalling pathways and the recruitment of the NADPH oxidase to the phagosome membrane (Lodge *et al.,* 2006). Some researchers have suggested uptake via the host cell apoptotic receptors prevents the activation of microbicidal responses (as well as pro-inflammatory cytokine release) and may be critical for promastigote survival in neutrophils (Gueirard *et al.,* 2007; van Zandbergen *et al.,* 2007). In contrast to promastigote stages, intracellular amastigotes downregulate the expression of LPG (and other surface macromolecules) and lack a conspicuous surface coat (Naderer *et al.,* 2004), although they retain a glycocalyx of parasite-synthesized GIPLs and host derived glycosphingolipids (McConville and Blackwell, 1991; Winter *et al.,* 1994; Naderer *et al.,* 2004). Leishmania amastigotes also synthesize inositol phosphoceramide (IPC) using sphingolipid bases salvaged from the host (Zhang *et al.,* 2005), and this abundant phospholipid may also complement the functions of the GIPLs. The plasma membrane of *Leishmania* amastigotes is unusual in several other respects. It contains relatively high levels of externally exposed phosphatidylserine that may provide a mechanism for entering host cells via apoptotic cell receptors without activating microbicidal processes or proinflammatory responses (Wanderley *et al.,* 2006).

Most species of *Leishmania* proliferate within individual, tight-fitting vacuoles, which have the characteristics of mature phagolysosomes and can fuse with late endocytic vesicles, phagosomes and autophagosomes, suggesting that there is a continuous flux of low molecular weight metabolites and macromolecules into the lumen of this compartment (Antoine *et al.,* 1998; Burchmore and Barrett, 2001). Phagolysosome lumen contains a variety of carbon sources and essential nutrients, but is hexose poor (McConville *et al.,* 2007; Opperdoes and Coombs, 2007). This requirement for hexose synthesis/uptake may be linked to the need for the pentose phosphate pathway required for regeneration of NADPH and precursors for RNA and DNA synthesis (Maugeri *et al.,* 2003) and/or the production of intracellular mannan (linear oligomers of b1–2mannose), the major short-term energy reserve of these parasites (Sernee *et al.,* 2006). *Leishmania* are auxotrophic for many amino acids and intracellular stages must scavenge their essential amino acid needs from the phagolysosome in order to use them in protein and polyamine biosynthesis as well as a major carbon source (McConville *et al.,* 2007). Amastigotes upregulate a large family of amino acid permeases for continious supply of free amino acids. They also upregulate the expression of lysosomal cysteine proteases and endocytose host proteins from the lumen or limiting membrane of the phagolysosome, providing an alternative source of amino acids (Besteiro *et al.,* 2007).

Leishmania must scavenge other essential nutrients (purines, haeme, vitamins) and cations (iron, magnesium) from the phagolysosome (Burchmore and Barrett, 2001; McConville *et al.,* 2007). Most of the metabolite transporters are proton-symporters that utilize the low pH of the phagolysosome to drive high affinity uptake (Burchmore and Barrett, 2001). *Leishmania* amastigotes express a high affinity ferrous (Fe2+) transporter, LIT1, which scavenges iron from the phagolysosome in competition to the host transporters (Huynh *et al.,* 2006).

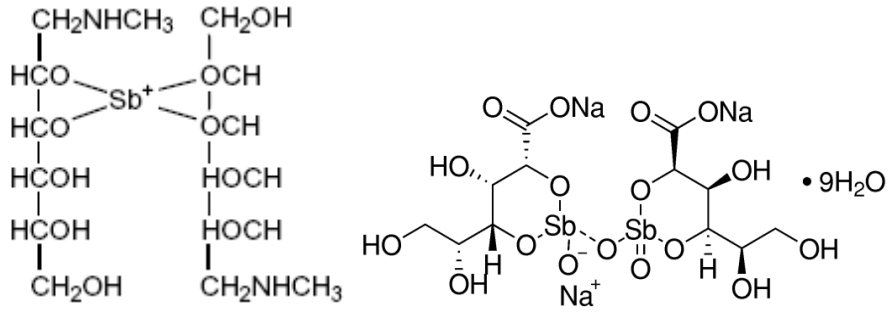
CHAPTER-2

REVIEW OF LITERATURE

2.1. Conventional therapy against VL

2.1.1. Agents effective by parenteral route

2.1.1.1. Pentavalent antimonials



A **B**

Fig.12 Chemical structure of Glucantime (A) and Pentostam (B).

Source: www.aventis.com and www.sigmaaldrich.com

N-methylglucamine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) have been used as a first line of treatment for VL since the 1940s (Fig.12). Antimony remains the therapeutic cornerstone in all regions except two: Bihar State, India and Southern Europe. In Bihar approximate 35% cure response has ended the usefulness of antimony and in southern Europe secondary resistance developed in patients who relapse (Sundar *et al.,* 2000a). Effective doses of Sodium stibogluconate and meglumine antimoniate are 20 mg/kg/day up to a maximum 1275 mg over 20 or 30 days given intramuscularly. Its intracellular reduced trivalent form is the active derivative that comes about through the alteration in parasite bioenergetic pathways and trypanthione inhibition (Ephros *et al.,* 1999; Wyllie *et al.,* 2004).

Antimonials are toxic drugs with frequent, sometimes life threatening adverse side effects, including cardiac arrhythmia and acute pancreatitis. Patients under the age of 2 or aged 45 or over with signs of advanced disease and/or severe malnutrition are at higher risk of death during antimonial therapy owing to drug toxicity, slowness of drug action, VL complications

or a combination of these factors (Chappuis *et al.*, 2007).

2.1.1.2. Pentamidine isothionate

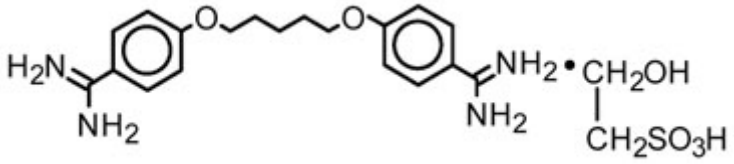


Fig. 13 Chemical structure of Pentamidine isothionate

Source: www.scielo.br.com

Pentamidine, an aromatic diamidine (Fig.13) has been previously used as a second line of treatment for VL but its precise mode of action has yet to be elucidated. Since, it is a competitive inhibitor of arginine transport and noncompetitively inhibits putrescine and spermidine, its leishmanicidal activity is possibly mediated via its influence on polyamine biosynthesis and the mitochondrial membrane potential. Pentamidine was initially proven to be useful in Sb^{v} resistant kala-azar cases in India (kala-azar unresponsive to antimonial were treated with pentamidines in a dose of 4 mg/kg body weight alternatively for 20 days) but the limiting factors were the expense and above all the unacceptable toxicity as it causes irreversible insulin dependent diabetes mellitus and death. Further, it's declining efficacy (as only about 70% patients could be cured), has led to its being totally abandoned in India (Sundar & Chatterjee, 2006).

2.1.1.3. Amphotericin-B & its formulations

Conventional Amphotericin B (fungizone®) is a macrolide polyene (Fig.14), charachterized by hydrophilic polyhydroxyl and hydrophobic polyene aspects. It is a powerful antileishmanial agent and is a first-line drug in India, where resistance to pentavalent antimonials is common.

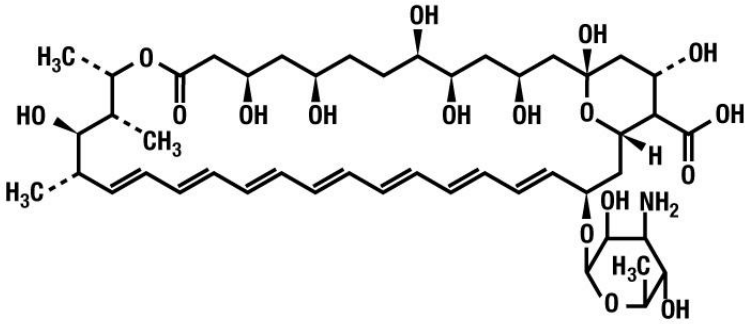


Fig. 14 Chemical structure of Amphotericin-B

Source: www.ambisome.com

The best amphotericin B regimen is 15 doses of 1 mg/kg on alternate days. This drug is characterized by infusion related side effects and renal toxicity. Moreover this drug is costly and requires a complicated regimen. Amphotericin-B binds to membrane ergosterol leading to the formation of pores, major constituent efflux and finally parasite cell lysis (Pape, 2008).

(i) Liposomal Amphotericin B

The liposomal amphotericin B formulation, AmBisome®, is registered treatment for visceral leishmaniasis (Meyerhoff, 1999), but use in VL endemic regions is limited by cost (US$2,800 per treatment). A dose of 5–20 mg/kg in 4–10 doses over 10–20 days, by intravenous route is optimized (Griensven *et al.,* 2010).With recent preferential pricing offered by the manufacturer to patients in the public sector in East Africa, it is possible that AmBisome® could become economically feasible for treatment, even in resource – poor countries (DNDi Annual report 2007- 2008).

(ii) Other commercial Amphotericin B

Lipid formulations have also been manufactured, namely an amphotericin B lipid complex given at a dose of 1-5 mg/kg per day(Abelcet®) and an amphotericin B colloidal dispersion given at daily dose of 1 mg/kg of body weight (Amphocil™) but their use against VL has not been as extensive as AmBisome® and they too are costly. Other re-formulations of

Amphotericin B formulations have been investigated against experimental VL but none have reached clinical development to date. Approaches to reduce cost include: (i) efficacy trials of single dose AmBisome treatment for VL, with 90 per cent cure rate reported to date, and (ii) the use of cheaper liposomal formulations, already tried for VL (Croft *et al.*, 2006a).

2.1.1.4. Paromomycin

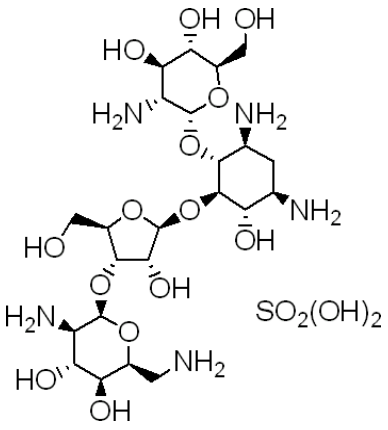


Fig. 15 Chemical structure of Paromomycin sulfate

Source: www.sigmaaldrich.com

Paromomycin, initially named aminosidine (Fig.15), was first isolated in the 1950s from filtrates of *Streptomyces krestomuceticus.* The spectrum of activity of paromomycin was found to encompass, like other aminoglycosides, most Gram-negative and many Gram-positive bacteria. The antibacterial action of paromomycin relates to its binding to the 30S ribosomal subunit, impairing protein synthesis. Unusually, paromomycin is also effective against some protozoa and cestodes and it is the only aminoglycoside with clinically important antileishmanial activity. Injectable paromomycin was extensively used as an antibiotic until Pharmacia (Milan, Italy) ceased production and marketing in the mid-1980s when cephalosporins and quinolones became popular antibiotics. Paromomycin in methylbenzethonium chloride ointment is used as a topical treatment for cutaneous leishmaniasis (CL) (*L.major*) in Israel (Leshcutan; Teva, Patah Tiqva, Israel). Paromomycin

is poorly absorbed after oral dosing and is still marketed as an oral treatment for amoebiasis and giardiasis and to decrease bacterial load in the gut in hepatic coma (Humatin; Parke-Davis, Morris Plains, NJ, USA). The antileishmanial properties of paromomycin were recognised by Kellina (1961) and were confirmed by Neal *et al.* (1968) and (1995). Two 'proof of concept' studies, by the Kenya Medical Research Institute (KEMRI) in Nairobi (Chunge *et al.,* 1990) and the Hospital for Tropical Diseases in London, showed excellent therapeutic activity against VL (Scott *et al.,* 1992).

Combination therapy using Paromomycin

The drug was being considered as a promising antileishmanial by the WHO/TDR. In 1991, a massive epidemic of VL occurred in western Upper Nile province in south Sudan. To combat this situation a humanitarian medical agency Médecins Sans Frontières (MSF) decided to evaluate a short-course combination of paromomycin plus SSG, which was by then also being studied in India by the WHO/TDR (Thakur *et al.,* 1992). Seaman *et al.,* (1993) conducted a randomised controlled trial of combination of paromomycin sulphate 15 mg/kg/day plus SSG 20 mg/kg/day, i.m. daily for 17 days on 200 Sudanese VL patients and were found to be as effective as SSG monotherapy, with a lower mortality.

Pharmacological class

The chemical name: *O*-2,6-diamino-2,6-dideoxy-β-l-idopyranosyl-(1→3)-*O*-β-d-ribofuranosyl-(1→5)-*O*-[2-amino-2-deoxy-α-d-glucopyranosyl-(1→4)]-2-deoxy streptamine sulfate.

The empirical formula: $C_{23}H_{45}N_5O_{14} \times H_2SO_4$.

The molecular weight of paromomycin base is 615.65 and sulfate is 896.86.

Generic names: aminosidine, catenulin, crestomycin, estomycin, hydroximycin, monomycin A, neomycin E and paucimycin.

Trade names: Gabbromicina, Gabromicina and Gabromycin for parenteral products, Gabboral, Kapseal, Humantin and Pargonyl for oral products and Leshcutan for the topical product.

3. Antileishmanial action

The leishmanicidal action of paromomycin is likely to be complex (Maarouf *et al.*, 1997) *L. donovani* may be killed through inhibition of parasite metabolism and mitochondrial respiration. In vitro studies have shown that paromomycin can effectively kill both promastigotes and amastigotes (Neal and Croft, 1984; Gebre-Hiwot *et al.*, 1992; Neal *et al.*, 1995; Maarouf *et al.*, 1998). The 50% effective dose (ED_{50}) of paromomycin against *L. donovani* amastigotes ranges from 10 μM to 50 μM. Data from mouse VL models and naturally infected dogs demonstrated that paromomycin is effective *in vivo* against *L. donovani/L. infantum* (Neal *et al.*, 1995; Buffet *et al.*, 1996; Gangneux *et al.*, 1997; Poli *et al.*, 1997; Vexenat *et al.*, 1998; Williams *et al.*, 1998). Several studies have been conducted of paromomycin, alone or in combination with other drugs, as treatment for VL. It should be noted that paromomycin sulfate 15 mg/kg ≈ paromomycin base 11 mg/kg. The most important study is that done by Sundar *et al.*, (2007), which led to the licensing of paromomycin for VL in India. Neal *et al.* (1995) showed paromomycin and SSG to be synergistic *in vitro* and additive in a mouse model of VL. In a study of interactions between antileishmanial drugs, Seifert and Croft (2006) reported miltefosine and paromomycin to be additive *in vitro* and synergistic in the mouse VL model. Several clinical trials have shown the combination of paromomycin and SSG to be more effective than monotherapy with either

drug (Chunge *et al.*, 1990; Seaman *et al.*, 1993; Thakur *et al.*, 2000).The largest report of paromomycin plus SSG is by Melaku *et al.* (2007).

In 2007, injectable paromomycin was licensed in India as an effective and well tolerated treatment for VL and was included in the WHO Model List of Essential Medicines for the treatment of VL. The drug costs for a 21-day course for a 35 kg VL patient (€4.19) make this the cheapest treatment available. Currently, the Drugs for Neglected Diseases Initiative (DND*i*) is conducting studies on paromomycin (as monotherapy and in combination) in VL in Africa, and the Institute of One World Health (iOWH) is conducting a Phase IV study in India. Because of the need to prevent the emergence of drug-resistant leishmaniasis in areas of human-to-human (anthroponotic) transmission (India and Africa), we consider that paromomycin should be used as part of combination therapy for VL, for example combined with SSG (where this is still effective) or liposomal amphotericin B (all regions). In addition, the distribution of paromomycin (like other drugs for leishmaniasis) should be well regulated, for example by being restricted to the public sector. These strategies should delay or prevent the emergence of resistance and ensure the longevity of paromomycin as a useful drug for VL.

2.1.2. Orally effective agents

2.1.2.1. Miltefosine

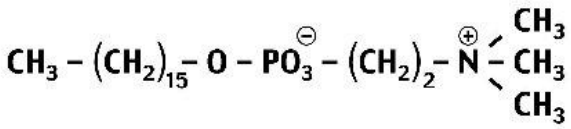


Fig. 16 Chemical structure of Miltefosine

Source: www.mpibpc.mpg.de.com

It was discovered in Goettingen by Prof. Hansjörg Eibl from the Max Planck Institute for biophysical chemistry, and Prof. Clemens Unger from Clinic for Tumor Biology at the Albert Ludwig University in Freiburg. The active substance miltefosine - its chemical name being hexadecylphosphocholine (HPC), an analogue of phosphatidylcholine (PC) - has a simple molecular structure (Fig.16). Croft and co-workers in the late 1980s demonstrated that miltefosine which was initially developed as an anticancer agent, quickly and effectively eliminated Leishmania promastigotes from culture. Attention to this compound led to preclinical and clinical studies conducted for leishmaniasis (Croft *et al.*, 1987). As a result, miltefosine has been registered for the treatment of visceral leishmaniasis in Germany and India, as well as for cutaneous and visceral leishmaniasis in Colombia.

Systematic name: 2-(hexadecoxy-oxido-phosphoryl) oxyethyl-trimethyl-azanium

Formula: $C_{21}H_{46}NO_4P$

Molecular Weight: 407.568 g/mol

Half life: 6 to 8 days

Identification of the antileishmanial potential of miltefosine started in the late 1980s (Croft *et al.,* 2003). The development of miltefosine for leishmaniasis began with studies on the metabolism of phospholipids in *L. donovani* promastigotes in 1982 (Hermann *et al.,* 1982), where it concluded that ethers of lysophospholipids (LPAs) such as 1-*O*-alkylglycerophosphocholine, 1-*O*-alkylglycerophosphoethanolamine and 1-*O*-hexadecyl-*sn*-glycerol were active and completely eliminated the parasites after less than 5 h of exposure to 25 µMol/l. Miltefosine was then administered orally to BALB/c mice infected with *L. donovani* and *L. infantum*, and 95% parasite elimination was achieved with a dosage of 20 mg/kg bodyweight (Kuhlencord *et al.,* 1992). The results stimulated the creation of a

clinical program for visceral leishmaniasis in India, where the first Phase I/II study was completed in 1997 (Sundar *et al.,* 1998). In 2000 and 2001, it was demonstrated that miltefosine was effective in immunodeficient animals in contrast with the lack of activity of sodium stibogluconate (Murray, 2000; Escobar *et al.,* 2001).

Mechanism of action

Although analogs of lipophospholipids (LPAs) were developed as anticancer agents, these compounds also have strong antiparasitic activity *in vitro* (Singh and Shivkumar, 2004). Due to its chemical nature as a lecithin analog, LPAs interact with a variety of sub cellular structures and biochemical pathways. In mammalian cells, LPA induces programmed cell death associated with the inhibition of phosphocholine biosynthesis. Due to its molecular structure, LPAs have been intensely investigates as potential inhibitors of the enzymes involved in the synthesis, degradation and modification of the lipid membrane (Wieder *et al.,* 1999). The metabolism of miltefosine was investigated in *L. mexicana* (Lux *et al.,* 2000) and it was found that these compounds inhibit the specific alkyl-specific acyl CoA acyltransferase, a key enzyme for ether lipid remodeling, which may exert an effect on cellular growth of parasites. PCD in Leishmania due to miltefosine is characterized by a typical apoptotic phenomenon, such as cellular shrinking, DNA fragmentation and phosphatidyl serine exposition, with preservation of the integrity of the plasmatic membrane, which may cause programmed cell death in these organisms and can explain the selective antiparasitic effects of such compounds *in vivo* (Paris *et al.,* 2004).

Metabolism & excretion

There is no interaction of miltefosine with cytochrome P450 metabolic enzymes, thus induction or inhibition of metabolism of other medications by these systems is not expected.

Phospholipase C metabolizes miltefosine and liberates choline, which is later used for the biosynthesis of acetylcholine or lecithin. Hexadecanol, the long-chain fatty alcohol that also results from phospholipase C activity, can be oxidized to palmitic acid and enter lipid biosynthesis or β-oxidation (Achterberg and Gercken, 1987). Patients with cancer demonstrated an increase in leukocytes and platelets during treatment with miltefosine (Pronk *et al.*, 1994). In rats, miltefosine at a dosage of 1-2 mg/kg during the early embryological development and during the formation of the organs has a risk of embryotoxicity, fetotoxicity and teratogenicity. Since there are no controlled studies with miltefosine in pregnant women, its use during pregnancy is strictly controlled. Furthermore, the mean half-life of miltefosine in humans is 1 week, the use of some type of effective contraception is recommended for 2 months after the last dose is taken.

2.1.2.2. Sitamaquine

Sitamaquine, an orally active 8-aminoquinoline analog (Fig.17) (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 in response to a pressing need for orally effective agents for VL, its effectiveness was validated in animal models.

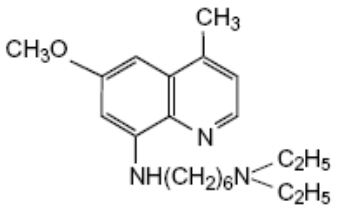


Fig.17 Chemical structure of Sitamaquine

Source: www.parasite.trends.com

Several small phase I or II clinical trials have been undertaken with limited success. The cure

rate for VL with sitamaquine in a Kenyan phase II study at a dose of 1 mg/kg/day for 28 days were 50 percent. Several years later, in a Brazilian phase II trial, the same dose of sitamaquine cured none of the four VL patients while a 2 mg/kg/day for 4 wk gave a maximum efficacy of 67% surprisingly, a linear correlation could not be sustained as increasing the dose to 2.5 mg/kg/day resulted in decreased efficacy concomitant with enhanced adverse effects such as nephropathy and methaemoglobinaemia. In a multicenter phase II trial in India, sitamaquine demonstrated excellent antileishmanial activity at a daily dose of 1.75 -2 mg/kg for 28 days. However, more studies are needed to evaluate some of the safety issues as this drug appears to have clinical efficacy that warrants further development. The mode of action is not known but could involved "futile redox cycling" as proposed for primaquine (Sundar & Chatterjee, 2006).

2.1.2.3. Azoles

Another promising approach for development of new antileishmanial agent is "Therapeutic Switching or "piggy-back therapy". Under this approach various azoles have been explored. Azoles (Ketoconazole, fluconazole, itraconazole, *etc*.) are essentially sterol bio-synthesis inhibitors.Their efficacy against *L. tropica* was first reported by Berman (1981). Azoles specifically block ergosterol synthesis and as the presence of ergosterol as a membrane component is shared between fungi and *Leishmania*, it accounts for many antifungal sterol biosynthesis inhibitors (SBIs) to also be leishmanicidal. Azoles have been shown to be active against a wide range of promastigotes and amastigotes. *Leishmania* species differ in their sensitivity to azoles as *L. donovani*, *L. braziliensis* and *L. amazonensis* promastigotes are more sensitive than *L. aethiopica*, *L. major*, *L. tropica* and *L. mexicana*. However, this analogy cannot be extrapolated to clinical studies. Both ketoconazole and fluconazole have undergone evaluation in VL in India. However, despite reports of the former's usefulness, their antileishmanial activity was not enough to induce clinical cure by themselves (Sundar &

Chatterjee, 2006).

2.1.2.4. Buparvaquone

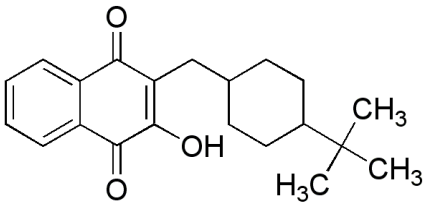


Fig.18 Chemical structure of Buparvaquone

Source: en.wikipedia.org

Buparvaquone (BPQ) is a hydroxynaphthoquinone (Fig.18) and marketed as Butalex® closely related to a well-known anti- infective drug, atovaquone. BPQ has been used as an i.m. injection for the treatment of theileriosis in cattle. For the first time Croft *et al.,* (1992) has tested BPQ against *L. donovani* infected BALB/c and observed a 62% suppression of hepatic amastigote burden. Researchers are looking forward to this drug as a promising antileishmanial agent as it has several physicochemical properties suitable for topical delivery (low molecular weight, low melting point etc). Attempts have been made to increase aqueous solubility and absorption, and in this context two phoaphate prodrugs have been found to show potential *in vitro* & *in vivo* antileishmanial activity against both visceral and cutaneous leishmaniasis (Ma¨ntyla *et al.,* 2004; Garnier *et al.,* 2007).

2.2. Drug Screening in Leishmania

2.2.1. Models used in drug screening

2.2.1.1. *In vitro* models

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 an ex-*in vivo* condition. Both these stages have been exploited for development of primary drug screening procedures.

Advantages:

(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.

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 defence system will not show any action. Therefore, *in vitro* testing at times may not be transferable to *in vivo* situation. Hence, there remains a glitch on the acceptability of *in vitro results*.

In 1986, 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

(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.

(i) Using promastigotes

The promastigotes grown in simple media have been used as test parasite to screen potential antileishmanial agents and the simplicity of this system accounts for its wide popularity. 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 1-2 × 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 *in-vitro* test with promastigote have very little value in animals (Peters *et al.*, 1983; Croft *et al.*, 2006b), the other conditions which reduce leishmanicidal action *in vitro* are the lower temperature (24°C) at which the culture normally grows, as opposed to the *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. Promastigotes assays are useful cytotoxicity indicators in bioassay-guided fractionation of plant products. Due to these problems, the use of promastigote for drug testing has been abandoned.

(ii) Using Amastigotes

Ideally to be efficient and exhaustive, a drug screening procedure requires conditions that tightly mimic the environment encountered by the target cell. For Leishmania, intracellular form of the parasite (amastigotes) might represent the ideal conditions. The role played by the host cell on drug mediated toxicity could be important.

(iii) Axenic Amastigotes

Jackson *et al*. (1989) have developed an *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 *in vitro*. Axenic amastigotes are obtained by subjecting promastigotes to pH change at 37^{o}C. A direct comparison of the drug susceptibility towards standard antileishmnial drugs, between

amastigotes and axenic amastigotes, demonstrates that the later express specific susceptibility to many if, not all the drug tested (Ephros *et al.,* 1999). Axenic amastigotes system for drug screening has been used by Callahan *et al*. 1997; Ephros *et al*. 1999; Sereno *et al*. 2007.

Screening against axenic amastigotes presents several advantages; (1) the test is directed against the relevant stage of parasite, (2) this stage is as easy to manipulate as the promastigote model, (3) quantification of drug activity is simple and often inexpensive. This can be achieved by using a cell counter (Ephros *et al.,* 1999) evaluating the viability of cell population with a MTT based method (Sereno and Lemesre, 1997; Ganguly *et al.,* 2005), by determining ornithine decarboxylase activity (Callahan *et al.,* 1997) or using a fluorescent dye like Propidium Iodide (PI) and fluorescence-activated-cell-sorter (FACS) (Sereno *et al.,* 2005). Since, past few years many Leishmania parasites expressing reporter genes have been selected and the capacity of some of them to be used in axenic amastigote drug screening protocol have been evaluated. Sereno *et al.* (2001) assessed luciferase expressing DNA transformed axenically grown *L. infantum* amastigotes and showed its use in high-throughput screening for new antileishmanial drugs. Rapid fluorescent assay using Alamar Blue for screening drugs on axenic amastigotes of *L.donovani* and *L.tropica* was also done by Shimonya and Jaffe (2008).

Disadvantages:

1. The assay is semi-predictive as it neither tests for penetration of the compound into the host cell nor for activity in the peculiar environment of the macrophage phagolysosome.
2. Axenic amastigotes may have different metabolic processes than intracellular amastigotes.
3. Screening with axenic amastigotes from clinical isolates is not possible because they require time to get adapted in the cultures.

(iv) Intracellular Amastigotes

The most widely used models for testing drugs against *Leishmania* species have involved either murine peritoneal macrophages or human-monocyte transformed macrophages (THP-1, U937, and HL-60) as host cells (Escobar *et al.,* 2002; Yardley *et al.,* 2005). In these differentiated non-dividing macrophages, the rate of amastigote division in host cells and drug activity can be clearly assessed. The activity of test drug is measured by either microscopically counting of percentage of infected cells or number of amastigotes per macrophage (Neal and Croft, 1984) or colorimetric or fluoremetric methods. The slow rate of division of *L. donovani* and *L. infantum* amastigotes in this model is a limitation. Assays that use dividing host cells must ensure that the confounding effects of drug activity on both parasite and host cell number are considered (Croft *et al.,* 2006a).

(v) In tumour macrophages

Mattock and Peters (1975) have extensively studied the effect of drugs on amastigotes of *L. donovani* in dog sarcoma cells. The disadvantage of this model is that conversely to macrophages, tumour cells are self multiplying. 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 *in vitro* might differ from *in vivo* (Berman *et al.,* 1985).

(vi) 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.

(vii) 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.

2.3.1.2. *In vivo* models

Animal models are expected to mimic the pathological features and immunological responses observed in humans when exposed to a variety of *Leishmania* spp. with different pathogenic characteristics. Many experimental models have been developed, each with specific features, but none accurately reproduces what happens in humans. For *in vivo* testing of new compounds several animal species have served as experimental host for VL. Important among them are BALB/c mice and Syrian golden hamster (primary tests), dogs (secondary tests) and monkeys viz., squirrel, vervet and Indian languor monkeys as tertiary screens. Animal models enable drug activity to be determined in relation to absorption (route of administration), distribution (different sites of infection), metabolism (pro-drugs, immuno-modulators), 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 (< 10 days) and have no indication of toxicity at the highest doses tested (100 mg/kg).

(i) Rodents Model

Several attempts were made in the past to use small rodents for *L. donovani* infection. These includes hamster (European, Chinese and Syrian); mouse (BALB/c, NMRI, DBA/1, C57BL/6) rat, mastomys, squirrel, gerbil *etc.* (Hommel *et al.,* 1995). A problem in all these models is the determination of drug activity upon necropsy and biopsy which has been dependent on microscopy to determine the level of infection.

(ii) Mouse model

Mouse model of leishmaniasis have been extensively used to study the pathogenesis of the disease and to test novel therapeutic and immuno-prophylactic agents (Murray *et al.,* 2003), where a relatively low amount of compound is required. Mice are susceptible to most strains and species of Leishmania in both non-cure and self cure models (Louis *et al.,* 2002; Courret *et al.,* 2003). For visceral leishmaniasis inbred strains of mice are widely used with susceptible, resistant and intermediate strains. Mice are infected intravenously or intracardially with $2x10^7$ *L.donovani* amastigotes dosed 7 days post infection for 5 consecutive days and sacrificed 3 days after the completion of treatment (day 14 post infection). Groups of mice are weighed before and after treatment, and the percent weight change is recorded. Impression smears are prepared from weighed livers followed by methanol fixation and stained with 10% Giemsa stain in water. The number of amastigotes per 500 liver cell nuclei are determined and multiplied by the liver weight (mg) to obtain Leishman-Donovan Units (Bradley and Kirkley, 1972). The percent inhibition was calculated for all drug-treated groups in relation to untreated group, and ED_{50} are calculated.

(iii) Hamster model

Although many hamster species are susceptible to *L. donovani* infection (Smyly and Young, 1924), the Syrian golden hamster *(M. 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).

Hamsters are infected intra-cardiacally. Many workers have chosen different days (day1, day3, and day 15) for initiation of drug testing. Duration of treatment and autopsy differ from researcher to researcher (Stauber *et al.,* 1958; Mikhail and Mansour, 1975; Hanson *et al.,* 1977). Among various techniques, method adopted by Beveridge's (1963) 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.) and it is, therefore, impossible to assess the delayed action of drugs. Bhatnagar *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, thus they are suitable for studying the sequential effects of drug in the model. This is more rational as it 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.

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 out bred hamsters. Dea-Ayuela *et al.,* (2007) have studied its suitability and established suitable immuno-biological parameters for *in vivo* testing of new antileishmanial compounds in the golden hamster model of visceral leishmaniasis. The clinico-pathological 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, hyper-gamma-globulinemia, and ultimately death (Gifawesen and Farrell, 1989). Major advantage is that repeated biopsy is possible to monitor pre- & post treatment infection status and all antileishmanials are active against liver as well as spleen parasites.de-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.

(iv) 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) and McKinney and Hendricks (1980) infected the African white tailed rat (*Mastomys albicandatus*) 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 *M. natalenis,* a multi-mammate rat as an experimental model for *L. donovani* and *L. major* and Dwivedi *et al.* (1983) successfully used this model.

(v) 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).

(vi) Non- Human Primate Model

Some of the observations made in rodent models might not be similar or relevant to human hosts due to distance in phylogeny. This leads to the development of a non human primate model for leishmaniasis which largely mimics the human situation. This would also

complement studies in other model systems. However, for financial and ethical reasons, the use of primates in biomedical research is limited. Studies involving these animals have therefore been tailored to solve questions that cannot be answered in other animals. Monkeys are normally the final experimental animals to be used in studies of the safety and efficacy of vaccines and drugs developed in other laboratory animals. Earlier efforts in establishing VL in New and Old World monkeys demonstrated that *Aotus trivirgatus* (owl monkeys) (Chapman *et al.,* 1983) and *Saimiri sciureus* (squirrel monkey) (Chapman and Hanson, 1981) developed an acute and fulminating, but short-lived, infection. Antileishmanial screening was performed in owl and squirrel monkeys. Old World monkeys such as Macaca sp. viz *M. mulatta, Macaca fascicularis* and *Macaca nemestrina,* and African vervet monkeys developed low and/or inconsistent infections (Hommel *et al.,* 1995). Attempts to establish VL in *Presbytis entellus* showed that this species was highly susceptible to single intravenous inoculation of hamster-spleen-derived *L. donovani* amastigotes, which invariably produced consistent and progressive acute fatal infection, leading to death between 110 to 150 days post-infection (p.i.). The infected animals presented all the clinico-immuno-pathological features as observed in human kala-azar (Anuradha *et al.,* 1992; Dube *et al.,* 1999). The Indian languor has also been used for preclinical evaluation of potential antileishmanial drugs and vaccine (Dube *et al.,* 1998, Misra *et al.,* 2001).

2.2.2. *In vitro* Techniques used in drug screening

2.2.2.1 Classical Methods of screening

Classical Screening methods are labour intensive and could not support automation. Initially Direct counting assays are used for evaluating drug activity towards intracellular amastigotes after methanol fixation and Giemsa staining on chamber slides (Berman and Wyler, 1980; Berman, 1984; Berman and Lee, 1984; Neal & Croft, 1984; Looker *et al.,* 1986; Gebre-Hiwot et al, 1992; Sereno *et al.,* 2007). The activity of the drug is determined microscopically by

the percentage of infected cells as well as the number of amastigotes per cell through examination of 100 -300 macrophages. IC_{50} could be determined by various methods, either by monitoring the reduction in the mean percentage of infected macrophages or by the mean reduction in the number of amastigotes per macrophage in drug treated cultures in relation to non treated cultures (Seifert and Croft, 2006). Counting cells is time consuming, labour intensive, subjective, and incompatible with high-throughput screening and may give inaccurate determination of IC_{50} since determination of the parasite viability through a staining procedure is difficult.

2.2.2.2. 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). Carmichael *et al.* (1987) and Alley *et al.* (1988) described modifications of Mosmann's procedure for *in vitro* assay of tumor cell response to chemotherapeutic agents. A major drawback with this method was the use of large quantities of potentially hazardous solution 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.*, 2001).

2.2.2.3. 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). Resazurin has been used since the 1950 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. It has been commercialized since 1993 as Alamar blue dye (O'Brien *et al.,* 2000) as a viability cell test. Alamar Blue is blue in its oxidized form. When reduced by bacteria or tissue culture cells, it changes to a bright pink colour 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 nonfluorescent resazurin to fluorescent resorufin has been used as a fluorometric indicator for the determination of cell viability. Viability measurement using resazurin is more or less similar to traditional tetrazolium salts (MTT, XTT) and [3H] thymidine assay techniques (Larson *et al.,* 1997). This technique 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).

2.2.2.4. Reporter gene Assays

The term reporter gene is used to define a gene with a readily measurable phenotype that can be distinguished easily over a background of endogenous proteins (Monte-Alegre *et al.*, 2006). Various recombinant parasites carrying a reporter gene either as an episomal copy or after its integration in a defined locus, generally the rDNA locus against leishmaniasis is currently available.

The use of reporter genes to monitor intracellular proliferation of micro-organisms has been effectively applied for bacteria (Changsen *et al.*, 2003) viruses, (Dorsky *et al.*, 1996) and other parasites (Buckner *et al.*, 1996). Such methods produce objective quantitative data, increase throughput, and decrease manual labour. A variety of reporter genes have been effectively used in biological screens including Green Fluorescent Protein (GFP), Chloramphenicol Acetyl Transferase (CAT), β-galactosidase, firefly luciferase, and alkaline phosphatase (Naylor, 1999).

2.2.2.5. GFP Fluorescent assay

GFP (Green fluorescent Protein) is an auto-fluorescent and stable protein, which originates from the jellyfish *Aequorea Victoria* (Prasher *et al.*, 1992; Tsien,1998; Sereno *et al.*, 2007).GFP based assays offer several advantages over other non-reporter or reporter gene-based- assays including greater simplicity, easier kinetic monitoring, low cost and enhance bio safety (Kain , 1999). Expression of GFP in several parasite species has been achieved and applied for drug evaluating studies (Sereno *et al.*, 2007).GFP leishmanial fusion protein have been synthesized for localization and trafficking analysis (Debrabant *et al.*, 2000).GFP expression in Leishmania was first achieved by Ha *et al.* (1996). Since then its expression by episomal vector has been carried out in several species of Leishmania (Singh and Dube, 2004; Mehta *et al.*, 2008) wherein the fluorescence intensity in parasites decreased with time in the absence of geniticin sulphate (antibiotic G 418), thereby necessitating its regular

addition (Roy *et al.*, 2000).Generally transfectants do not express sufficient levels of fluorescence for spectrofluorometric measurement on micro plate. To overcome this kind of problem Chan *et al.* (2003) have developed a spectrofluorometric assay wherein multimeric form of the GFP was engineered and expressed in *L. amazonensis* promastigotes. As expected, parasites expressing the multimeric GFP form bear fluorescence quantifiable in 96 wells with spectrofluorometric analysis. The integration of the GFP gene downstream of the 18 S rRNA gene promoters was done by Singh *et al.* (2009) at the ribosomal locus within the genome of the parasite, which also represents a valuable tool for drug screening in macrophages.

2.2.2.6. β-galactosidase

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. Promastigotes of Leishmania expressing β-galactosidase were selected and their use in drug screening procedures evaluated by Okuno *et al.* (2003). β-galactosidase presents the advantage that colorimetric detection can be performed. However, some commonly cited drawbacks of β-galactosidase include its large size (the monomer is 116 kDa), sensibility, the endogenous expression of β-galactosidase by some mammalian cell types including macrophages.Also coloured drugs can interfere the results and some compounds may alter the effects of enzyme or *vice-versa* precluding the use of these parasites for drug screening purpose (Campbell, 2005; Buckner and Wilson, 2005).

2.2.2.7. β –Lactamase

Buckner and Wilson, 2005 developed two species of Leishmania: *L. major* and *L. amazonensis* expressing β-lactamase. Overall, the results obtained demonstrate that this methodology could be valuable for drug screening procedures (Zlokearnik *et al.*, 1998). A simple colorimetric β-lactamase assay for quantifying Leishmania amastigotes grown in micotiter plates has been reported by Mandal *et al.* (2009). The β-lactamase gene was integrated into rRNA region of the genome, there by allowing for high-level stable expression of the enzyme. Both visceral Leishmaniasis and post- kala azar dermal leishmaniasis isolates were transfected with β-lactamase gene. Quantification was done by a colorimetric readout with CENTA ^{TM} β-lactamase as substrate and with an optical density plate reader. This methodology could be a valuable high-throughput screening assay for checking efficacy of anti-leismanial drugs in the clinical isolates (Mandal *et al.*, 2009).

2.2.2.8. Luciferase assay

The luciferase reporter gene technology is being widely used to monitor cell proliferation under *in vitro* culture systems, to monitor 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. The only drawback of this system is the use of expensive substrate and lyses of cells to detect the signal.

Various species of parasites expressing luciferase were recently developed and their susceptibility towards classical antileishmanial agents investigated (Roy *et al.*, 2000; Sereno

et al., 2001; Ashutosh *et al.*, 2005). Drug discovery facilities at Central Drug Research Institute (CDRI), Lucknow have developed *L. donovani* cell lines expressing firefly luciferase reporter gene (luc.) as a part of episomal vector and established suitability of these cell lines for *in vitro* screening of antileishmanial agents (Ashutosh *et al.*,2005). This system has been adapted to evaluate compounds in a 96 well micro plate format and is being employed (Pandey *et al.*, 2006; Gupta *et al.*, 2007; Sunduru *et al.*, 2009) for primary screening of novel synthetic compounds and marine extracts (Inhouse and MoES Project) and also for optimization of leads under DNDi supported consortium.

The main advantages of this technology are numerous and include the high sensitivity of the test and the absence of background activity in the host cell. Recently, a refined work performed by Lang and co-workers demonstrated that *L. amazonensis* parasites expressing firefly luciferase could be used to monitor Leishmania infection in real time, through imaging analysis. They have also tested various antileishmanial compounds and have followed their efficacy in live cells by using imaging (Lang *et al.*, 2005). The advantages of this methodology rely on the capacity to perform experiments on live cells, making the analysis faster and more accurate since viability of both the parasites and the host cells is monitored.

2.2.2.9. Multiplexing

A versatile methodology that allows for multiple quantifications of drug toxicity against both the host cells and the intracellular amastigotes (multiplexing) could represent a useful tool in the field of parasite pharmacology. By using this method we can simultaneously gather information of the viability of the host cell and the parasite, working with a combination of parasites and macrophages expressing different reporter. To achieve this goal, reporters must use distinguishable signal from each other and compatible chemistry, like fluorophores emitting different wavelengths. Currently, there have been a growing number of examples using luminescence for multiplexing either in combination with: (i) other luminescent signals,

(ii) fluorescence (iii) β –galactosidase assay (Grover *et al.,* 2003; Young *et al.,* 2004). Such methods could also help to directly compare experiments since the results are expressed as a ratio of the output signal emitted by the host cell on the one emitted by the parasites. The usefulness of these approaches for drug screening has to be evaluated on intracellular parasites like Leishmania or *T. cruzi* (Sereno *et al.*, 2007).

2.2.2.10. High- content/ high- throughput screening for the discovery of new anti-leishmanial drugs

High content screening combined with high-throughput screening (HCS/HTS) and automated image analysis considerably speed up the drug discovery process and allow for the screening of a large number of compounds in complex phenotypic assays involving whole cells. Siqueira-Neto *et al.* (2009) at Pasteur Institute, Korea with the aim to develop new anti-leishmanials, have adapted *L. donovani* intra-macrophagic amastigote culture to a HCS/HTS assay as a cellular model for Leishmaniasis. They optimized infection of the human macrophage cell line THP-1 by *L.donovani* metacyclic promastigotes in order to obtain very high yields of amastigotes-infected macrophages. Infection rates were assessed by an in house built algorithm for the counting of *L. donovani* infected macrophages and the number of amastigotes per macrophage. Countings were normalized in relation to negative controls, thus obtaining confident and unbiased data on the leishmanicidal activity of compounds. The algorithm also simultaneously evaluated the cytotoxicity of compounds by analyzing the viability of macrophages, thus eliminating false positive conditions. This assay was validated in the high- throughput format with 15 thousand points, including positive and negative controls as well as non infected macrophages. Currently, they are screening 200,000 drug-like small compounds from a chemically diverse library, using the assay and the algorithm described above.

2.2.3. *In vivo* Techniques used in drug screening

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.

2.2.3.1. 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. 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 day1 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. 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.

2.2.3.2. Mikhail and Mansour's technique

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.

2.2.3.3. Hanson's technique

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 (im), intra - peritoneal (ip) or oral (po). 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. 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 .

$$\textbf{Glucantime index (G.I.)} = \frac{\textbf{SD}_{90}\textbf{ for Glucantime}}{\textbf{SD}_{90}\textbf{ for test compound}}$$

2.2.3.4. Beveridge's technique

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 post treatment, thus allowing sufficient time for drug action.

2.2.3.5. Technique of Guru *et al.*

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 post treatment (pt) in addition to on day 7 and day 14 pt 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. In Stauber's and Hanson's techniques, the assessment is based on the effect of a potential drug on day 1 - 3 post infection (pi) 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 pi, the treated and control animals were sacrificed on day 60 pi. 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 pt). 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 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.

2.2.3.6. Real time GFP imaging of a murine leishmaniasis model

Mehta *et al.*(2008) used a Leishmania mutant episomally transfected with enhanced green fluorescent protein, enabling *in vivo* real time whole body fluorescence imaging, to follow the progression of Leishmania infection in parasitized tissues. Fluorescence correlated with the number of Leishmania parasites in the tissue and demonstrated the real-time efficacy of a therapeutic vaccine. This approach provides several substantial advantages over currently available animal model systems for the in vivo study of immuno-pathogenesis, prevention, and therapy of leishmaniasis. These include improvements in sensitivity and the ability to acquire real-time data on progression and spread of the infection.

2.3. Molecular mechanisms involve in parasite invasion (Apoptosis and its effects)

Apoptosis was first described by Kerr, Wyllie and Currie in the early 1970s and is defined by the morphologic appearance of the dying cell, which includes blebbing, chromatin condensation, nuclear fragmentation, rounding, and cell shrinkage. Biochemical features associated with apoptosis include high molecular weight DNA fragmentation into an oligonucleosomal ladder, phosphatidyl serine externalization, drop in mitochondrial membrane potential and proteolytic cleavage of a number of intracellular substrates. Survival of most of the cells requires continuous stimulation or positive signals from other cells and, for many, continued adhesion to the surface on which they are growing. Apoptosis can be induced due to withdrawal of these positive signals. Some examples of positive signals are growth factors for neurons and Interleukin-2 (an essential factor for the mitosis of lymphocytes). On the other hand increased receipt of negative signals can also result in

apoptosis for example increased levels of oxidants within the cell, damage to DNA by these oxidants or other agents like, ultraviolet light ,x-rays, chemotherapeutic drugs, accumulation of proteins that failed to fold properly into their proper tertiary structure ,molecules that bind to specific receptors (death receptors) on the cell surface and signal the cell to begin the apoptosis program. These death activators include: Tumor necrosis factor-alpha (TNF-α) that binds to the TNF receptor; Lymphotoxin (also known as TNF-β) that also binds to the TNF receptor; Fas ligand (FasL), a molecule that binds to a cell-surface receptor named Fas (also called CD95). There are 3 different mechanisms by which a cell commits suicide by apoptosis.

1. One generated by signals arising within the cell (Intrinsic mechanism)
2. Another triggered by death activators binding to receptors at the cell surface(Extrinsic mechanism) viz.TNF-α, Lymphotoxin , Fas ligand (FasL)
3. A third that may be triggered by dangerous reactive oxygen species.

2.3.1. Apoptosis triggered by internal signals: the intrinsic or mitochondrial pathway

In a healthy cell, the outer membranes of its mitochondria display the protein Bcl-2 on their surface which inhibits apoptosis. Internal damage to the cell (e.g., from reactive oxygen species) causes a related protein, Bax, to migrate to the surface of the mitochondria where it inhibits the protective effect of Bcl-2 and inserts itself into the outer mitochondrial membrane punching holes in it and causing cytochrome c to leak out. The released cytochrome c binds to the protein Apaf-1 ("apoptotic protease activating factor-1"). Using the energy provided by ATP, these complexes aggregate to form apoptosomes. The apoptosomes bind to and activate caspase-9. Caspase-9 is one of a family of over a dozen caspases. They are all proteases. They get their name because they cleave proteins mostly at aspartic acid (Asp) residues. Caspase-9 activates other caspases (caspase-3 and -7). The activation of these "executioner"

caspases creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation) which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell (Fig.19).

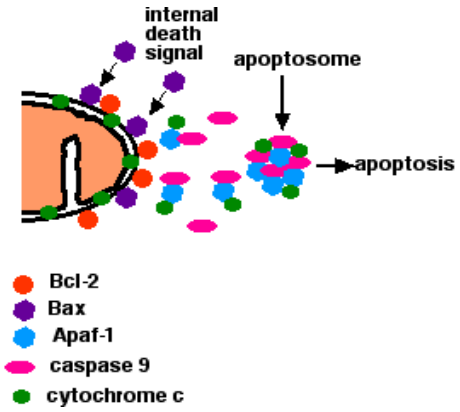


Fig.19 Components of the intrinsic or mitochondrial pathway

Source: www. users.rcn.com

2.3.2. Apoptosis triggered by external signals: the extrinsic or death receptor pathway

Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. Their binding with the complementary death activator (FasL and TNF respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8, which like caspase 9 initiates a cascade of caspase activation leading to phagocytosis of the cell (Fig.20).

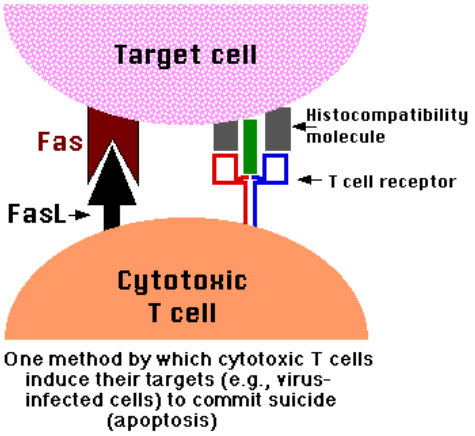


Fig.20 Components of the extrinsic or death receptor pathway

Source: www. users.rcn.com

2.3.3. Apoptosis-Inducing Factor (AIF)

Apoptosis-inducing factor (AIF) is a protein that is normally located in the intermembrane space of mitochondria. When the cell receives a signal telling it that it is time to die, AIF is released from the mitochondria (like the release of cytochrome c in the first pathway); migrates into the nucleus; binds to DNA, which triggers the destruction of the DNA and cell death.

2.3.4. Apoptosis in Leishmania

In the context of *Leishmania* spp., a unicellular eukaryote responsible for causing leishmaniases, the process of apoptosis is important for successful survival. The flagellated promastigote form of the parasite resides in the midgut of the insect vector (the female sandfly) and at this niche; the cell fittest to survive to pass onto the pharynx of the fly is selected by eliminating unfit cells through apoptosis (van Zandbergen *et al.*, 2006, 2007). Within the mammalian host, inside the macrophage, apoptosis is necessary to regulate cell numbers and to minimize immune reactions. *L. donovani* shows typical features of apoptotic death like cell shrinkage, nuclear condensation and DNA fragmentation. Agents capable of

precipitating apoptosis in this parasite include antileishmanial drugs like antimony, amphotericin B, pentamidine and miltefosine. Other agents like heat shock, treatment with staurosporine, knocking out centrin gene also cause apoptosis of the parasites. A pivotal role in cellular apoptosis is played by the single mitochondrion of *Leishmania* spp., where a fall or increase in mitochondrial potential leads to cell death by apoptosis.

2.3.4.1. Characteristic features of apoptosis analysed during study

Apoptotic cells are characterized by morphological markers such as cell shrinkage, posphatidylserine (PS) exposure, mitochondrial membrane depolarization, DNA fragmentation, membrane blebbing and packaging of cell contents into apoptotic bodies.

(i) Phosphatidylserine exposure and its detection

PS is a structural phospholipid normally localized on the inner surface of the plasma membrane. Apoptotic cells lose membrane asymmetry, and PS is exposed on the outer leaflet of the plasma membrane. Recognition of PS on the surface of apoptotic cells drives phagocytes to internalize these cells (Fadok *et al.,* 1992; van den Eijnde *et al.,* 1998). Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

(ii) Mitochondrial membrane potential loss and its detection

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, there is a collapse in the electrochemical gradient (referred to as $\Delta\Psi$) across the mitochondrial membrane. The collapse is thought to occur through the

formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm, which promotes the activation of caspases, which are directly responsible for apoptosis (Luo *et al.,* 1998; Narita *et al.,* 1998; Desagher *et al.,* 1999; Basanez, *et al.,* 1999). A unique fluorescent cationic dye, JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'- tetraethyl benzimidazolyl carbocyanine iodide), is used to signal the loss of mitochondrial membrane potential (Smiley *et al.,* 1991). In healthy non-apoptotic cells, the dye stains the mitochondria bright red (Cossarizza *et al.,* 1993).The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, J-aggregates form, which become fluorescent red. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells which show red and green fluorescence. The aggregate red form has absorption /emission maxima of 585/590 nm (Smiley *et al.,* 1991). The green monomeric form has absorption / emission maxima of 510/527 nm. The JC-1 monomers and aggregates give strong positive signals, capable of yielding both qualitative and quantitative results. Detection methods include flow cytometry, fluorescence microscopy, and a fluorescent 96-well plate reader format.

2.4. Host immune responses during Leishmania infection

Immunological responses against parasitic infections depend on the location of the parasite within the host. Those parasites which have the life cycle stages in which they are free within the blood stream, humoral immunity is most effective. Many of the parasites are capable of

intracellular growth, during which cell mediated immune reactions are effective in host defence.

Leishmania parasite deviously deactivates these effectors functions of the macrophage, thus allowing it to survive and persist within the macrophage. To achieve this, parasite employs multiple strategies leading to disease progression. It causes alteration in component of complement system, suppression of antileishmanial molecules and modulation in cytokine production which lead to occurrence of Th2 type of response. Secondly, parasite makes its entry to that host cells which lack leishmanicidal effecter mechanisms. This is helpful in providing protection against antileishmanial products secreted by host immunological cells. Besides this, suppression of MHC class II expression and sequestering of amastigote antigen presentation are some more strategies applied by Leishmania parasite for establishment of infection (Awasthi *et al.*, 2004, Santar'em *et al.*, 2007, Ali Ouaissi, 2007).

2.4.1. Cell Mediated Immune response

Leishmania is an intracellular parasite so CMI constitutes an important role in host defence against Leishmania infection. However initially, at the time of deposition of parasite by the sand fly bite on host skin, complement system i.e., humoral branch of immunity play very important role. In later stages Leishmania promastigotes bind to the surface molecules like complement receptor 1 & 3(CR1 & 3) and mannose receptor of macrophages by their ligands Lipophosphoglycan (LPG), gp 63 etc. They are then taken up by mononuclear phagocytes. Parasites attach to macrophage in a random non oriented manner.

Uptake of promastigotes occurs via circumferential engulfment by pseudopods, resulting in a strictly phagolysosomal localization of Leishmania. After internalization of the organism into phagosomes, secondary lysosomes are fused to form the complete parasitophorous vacuole. These foreign particles (Leishmania parasite) are destroyed by proteolytic enzymes

and by the production of reactive oxygen species. T cells, a type of lymphocyte (white blood cell), have an important role in the body's immune system. When a T cell encounters an invading foreign agent it begins to divide, forming four different types of T cell, each with a different function (Fig.21). **Killer T cells** destroy foreign agents by lysis. **Helper T cells** activate more killer T cells and also stimulate B cells to begin antibody production (unlike B cells, T cells do not produce antibodies to destroy invading pathogens). **Suppressor T cells** protect healthy cells from this attack and **memory T cells** persist in the bloodstream to guard against re-infection. T cells being an important component of CMI play very important role in immunity against Leishmania infection. Data from different experiments establish the role of specific $CD4^+$ T cells during the course of infection. Resistance and susceptibility to the disease depend on the Th1 or Th2 type of the immune response. It has been well documented that T cells can differentiate either in Th1 or Th2 type of effectors cells and this plasticity of differentiation depends chiefly on the priming during differentiation. Cytokines like IL4 and IL10 induce Th2 type of response and make the host susceptible to the disease whereas cytokine IL12 (produced by macrophages), IFN-γ (produced by NK cells) induce Th1 differentiation which is helpful in clearance of infection.

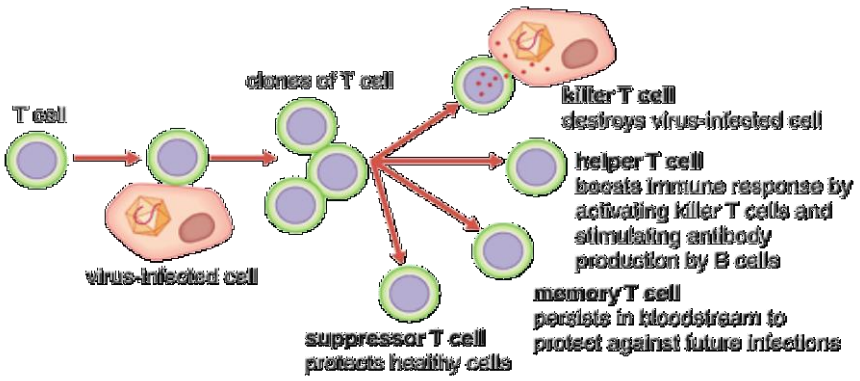


Fig.21 T cell Differentiation

Source: www.wikipedia.com

B cells, a type of lymphocyte (white blood cell), have an important role in the body's immune system. When a B cell encounters an invading bacterium it starts to divide, forming two different types of cell (Fig.22). One type is a clone of itself that begins to produce antibodies to fight the infection; the other is a memory cell that will persist in the bloodstream, ready to produce antibodies when re-infection occurs.

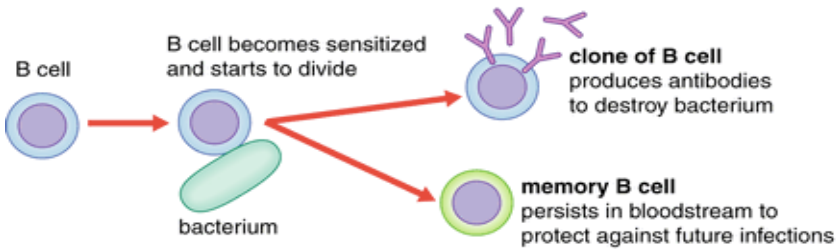


Fig.22 B cells Differentiation

Source: Source: www.wikipedia.com

2.4.2. Lymphocyte proliferation analysis

Lymphocyte proliferation normally occurs early in an immune response. Lymphocyte Transportation Assays tests the integrity of the early proliferative response using either nonspecific mitogens or specific antigens to induce blastogenesis. Antigen induced lymphocyte proliferation also correlates with previous exposure and acquisition of cellular immunity. Lymphocyte Transformation Tests evaluate lymphocyte competence using *in vitro* tests to assess the ability of the lymphocytes to proliferate and to recognize and respond to antigens. Two types of lymphocyte transformation tests, mitogens assay and antigen assay are usually performed. The mitogen assay, involve use of nonspecific plant lectins, evaluates the mitotic response of T and B lymphocytes to a foreign antigen. In the mitogen assay, a purified culture of lymphocytes is incubated with a nonspecific mitogen for 72 hours. The culture is then pulse-labeled with tritiated thymidine and can be measured by a liquid scintillation spectrophotometer in counts per minute, which parallels the rate of mitosis. Lymphocyte responsiveness or the extent of mitosis is then reported as a stimulation index,

determined by dividing the counts per minute of the stimulated culture by the counts per minute of a control (unstimulated) culture. The antigen assay uses specific antigens, such as purified protein derivative (PPD), Candida, mumps, tetanus toxoid and streptokinase, to stimulate lymphocyte transformation. After incubation of 4 ½ to 7 days, transformation is measured by the same method used in the mitogen assay. In the mitogen and antigen assays, a low stimulation index or unresponsiveness indicates a suppressed or defective immune system. Lymphocyte Transformation Tests are used for many reasons. Some uses are considered not medically necessary, such as its use as a screening test and to monitor cancer, occupational exposure to dust and other antigens, and other environmental antigens and mitogens.

2.4.3. Generation of toxic oxygen and nitrogen metabolites in response to parasite infections (Fig.23)

(i) Reactive oxygen species (ROS) are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural by-product of the normal metabolism of oxygen and have important roles in cell signalling. However, during times of environmental stress (such as for example, UV or heat exposure) ROS levels can increase dramatically, which results in significant damage to cell structures. This cumulates into a situation known as oxidative stress. They are also generated by exogenous sources such as ionizing radiation (Muller, 2000).

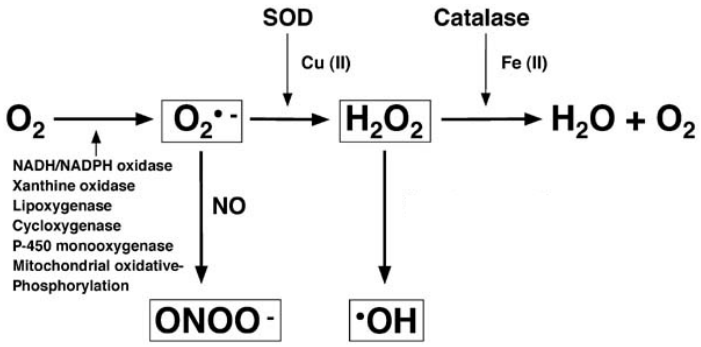


Fig: 23 Generation of Reactive Oxygen and nitrogen Species

Source: www.chinaphar.com

(ii) Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from nitric oxide (NO·) produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2). NOS2 is expressed primarily in macrophages after induction by cytokines and microbial products, notably interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) (Iovine *et al.,* 2008). RNS are produced in animals through the reaction of nitric oxide (NO·) with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (Squadritoa & William, 1998; Wolf, 2002). They act together with reactive oxygen species (ROS) to carry out detrimental effects on cells. Therefore, these two species are often collectively referred to as ROS/RNS. Reactive nitrogen species are also continuously produced in plants as by-products of aerobic metabolism or in response to stress (Pauly *et al.,* 2006).

The macrophage is armed with antimicrobial mechanisms that intracellular organisms must evade to survive. During leishmaniasis the microbicidal interactions between parasite and host cells occur in two stages. First, during initial phagocytosis of promastigotes the macrophage can undergo an oxidative response stimulated by the phagocytosis event. Second, once infection with amastigotes is established, the quiescent macrophage can be activated to potentially kill intracellular Leishmania. Efficient evasion of toxic microbicidal molecules produced at each stage of infection is important for Leishmania to be able to

initiate and maintain host cell infection. Two important macrophage-derived oxidants have been identified as critical in controlling Leishmania infection viz. superoxide ion and nitric oxide ion. During the first stages of infection superoxide (O_2^-) is produced as part of the respiratory burst of human and murine macrophages in response to phagocytosis (Murray, 1982; Channon *et al.,* 1984). O_2^- production is catalyzed by the NADPH oxidase, a heme-containing cytochrome that contains cytosolic and membrane-bound components. Once assembled the oxidase transfers an electron from NADPH to molecular oxygen, producing O_2^-. Leishmania promastigotes have shown to be susceptible for killing by exposure to O_2^- and hydroxyl radical (OH^-) generated from H_2O_2 (Zarley *et al.,* 1991; Miller *et al.,* 2000). A second anti-leishmanial oxidant produced by macrophages is NO (Cunha *et al.,* 1993; Evans *et al.,* 1993; Diefenbach *et al.,* 1998). Unlike O_2^-, which is a generated during phagocytosis of the parasite, NO is generated after macrophage activation by IFN-γ and TNF-α and is most relevant for the killing of established intracellular amastigotes. NO^- inhibitors such as *N*-G-monomethyl-L-arginine (L-NMMA) lead to an increase in amastigote survival and replication in murine macrophages (Liew *et al.,* 1990). Although there is strong evidence that NO^- plays an important role in murine leishmaniasis, it remains controversial whether NO^- plays a role in the antileishmanial responses of human macrophages (Murray & Teitelbaum, 1992; Vouldoukis *et al.,* 1995). NO^- was reported to participate in the killing of *L. major* by human macrophages that are stimulated through the low affinity Fce receptor, CD23, and IFN-γ (Vouldoukis *et al.,* 1995). Reactive oxygen and nitrogen species mediate cytotoxicity through alterations in protein; lipid and nucleic acid structure in function with resultant disruption of cellular homeostatic mechanisms. The figure shows one common series of reactions. A hydroxyl radical removes a hydrogen atom from one of the carbon atoms in the fatty acid chain and forming a molecule of water and leaving carbon atom with an unpaired electron

(radical). This radical react with oxygen forming peroxyl radical. This in turn remove hydrogen atom from nearby side chain creating a new radical.

(iii) Detection of ROS, RNS and H_2O_2

Bass *et al.* (1983) first applied flow cytometric analysis to study the generation of ROS by PECs. DCFH (2, 7-dichlorofluorescein diacetate) is a small non-polar and non-fluorescent molecule. It diffuses into the cells where it is enzymatically deacetylated by intracellular esterase to a polar but non-fluorescent compound, 2, 7-dichlorofluorescein (DCF).DCFH then get trapped within the cell where it remain stable for few hours. When PEC oxidative burst get activated, DCFH is oxidized to DCF in the presence of generated ROS. DCF emits a fluorescent signal that is measured with excitation at 498 nm and emission at 522 nm by using flow cytometer. The resultant fluorescence of the product DCF is linearly related to activity of the respiratory burst and appears to be mediated by ROS and H_2O_2 generated from stimulated PECs.For the fluorimetric detection of nitric oxide we use diaminofluorescein 2 diacetate (DAF2DA) which is non fluorescent cell permeable dye. DAF2DA diffuses into cells and tissue where non-specific esterase hydrolyzes the diacetate residue and trap DAF-2 within the intracellular space. NO produced react with DAF-2 and convert it to a highly fluorescent product DAF-2 Triazole (DAF-2T).Fluorescence given by various groups is compared with decrease in fluorescence when treated with inhibitors. Difference in fluorescence justifies the more commonly used killing mechanism. Inhibitors like NEM & PTX, L-NAME, NaN_3 inhibit NADP-oxidase, NOS and catalase respectively. Use of these inhibitors blocks the oxidation processes and thus there is less generation of fluorescence. These inhibitors are added in equal concentration to PECs of drug treated and control animals (Tarpey *et al.,* 2004).

2.4.4. Phagocytosis

Phagocytosis is an important weapon in the arsenal of the innate immune system. It is performed by 'professional phagocytes' and targets microbes into a cellular compartment where they can be killed. However, *Leishmania* spp. has developed mechanisms to subvert the microbicidal activity of macrophages. Following are the suggested mechanisms for phagocytosis of Leishmania parasite:

(i) Classical "Zipper" type phagocytosis: Promastigote uptake occurs by the classical 'zipper' type of phagocytosis, as well as 'coiling'phagocytosis. In the zipper mechanism, the initial attachment of the microbe to receptors on the phagocyte triggers the recruitment of additional receptors from the surrounding membrane, with a concurrent rearrangement of the cytoskeleton. This enables the extension of a pseudopod, which advances along the organism like a zipper, engulfing it into a phagosome (Rittig *et al.,* 1998, 2000). The early, plasma membrane-linked events of zipper phagocytosis are i) the attachment of the particle to the phagocytic receptor, ii) the engulfment of the adherent particle by protrusions extending from the cell surface, and iii) its internalization, combining the formation of a sealed vacuole and its budding off the restored cell membrane; this separated phagosome undergoes iv) further intracellular processing, eventually turning into a phagolysosome upon participation in the general endocytic trafficking of the host cell (Rittig *et al.,* 1999).

(ii) Coiling phagocytosis: It involves asymmetrical occurrence of pseudopodia coils and other multilayered pseudopod stacks.It has been suggested as an additional mechanism for parasite uptake (Rittig *et al.,* 1998, 2000). Complement receptor (CR) 1 and CR3 play major roles in both processes, and might act in concert to facilitate parasite binding and uptake. Uptake by coiling phagocytosis could target the organism to a cytoplasmic compartment and affect their survival (Bogdan and Rollinghoff, 1999). The interaction of the parasite with CRs occurs in three ways: (1) in the presence of serum by activating the complement component

C3 and binding through the C3bi fragment of complement to CR3; (Rittig *et al.*, 2000) through the serum-independent binding of the surface protease gp63 to CR3 (Rittig *et al.*, 1998); and through the binding of parasite lipophosphoglycan to the lectin-like site on CR3 and to CR1 (Handman, 1999). Engagement of the CRs does not trigger the respiratory burst and, in fact, opsonization by complement improves parasite survival (Mosser, *et al.*, 1987; Mosser and Edelson, 1987). The CR4, fibronectin receptor, mannose receptor and the advanced glycosylation end-product receptor have also been implicated in invasion (Alexander and Russell, 1992). It is likely that multiple receptor-ligand interactions occur simultaneously, depending on the activation state of the macrophage. Moreover, in view of the diverse host range of Leishmania, they may use different receptors for gaining access to different hosts.

CHAPTER-3

COMBINATION THERAPY FOR LEISHMANIASIS

3.1 Introduction

Combination therapy for the treatment of visceral leishmaniasis has increasingly been advocated as a way to increase treatment efficacy and tolerance, to reduce treatment duration and cost, and to limit the emergence of drug resistance. There are several reasons why consensus has grown over the past few years towards the use of combination regimens in visceral leishmaniasis (Bryceson, 2001; Alvar *et al.,* 2006; Croft *et al.,* 2006; Den Boer and Davidson, 2006; Singh *et al.,* 2006b; Sundar and Olliaro, 2007). First, combining drugs from different chemical classes could reduce treatment duration or total drug doses, resulting in fewer toxic effects, higher compliance, and less burden on the health system. This could also reduce the overall costs (direct and indirect) and provide a more cost-effective option. Increasing reports of treatment failure with pentavalent antimonials from the Indian subcontinent have raised the issue of acquired drug resistance (Lira *et al.,* 1999; Sundar, 2001; Croft *et al.,* 2006). This concern now extends to miltefosine, because of its long half-life and susceptibility to develop resistance with a single point mutation (Sundar and Murray, 2005; Perez-Victoria *et al.,* 2006; Seifert *et al.,* 2007). Finally, combination therapy could improve treatment efficacy for complicated cases, such as patients co-infected with HIV, for whom treatment outcomes with monotherapy have been consistently poor (Alvar *et al.,* 2006). The use of combinations to combat resistance has been well rehearsed in antimalarials. If a target enzyme has a mutation rate of 10^{-7}, the chances of resistance to a single agent developing is high, but the likelihood of developing resistance to two compounds with different targets is very low. Studies to identify such combinations are new for leishmaniasis. Studies are under way to examine interactions between miltefosine with other antileishmanials to identify suitable combinations (Croft *et al.,* 2006).

3.2 Preclinical data on combination therapy

An early study looked at interactions between sodium stibogluconate and paromomycin (Neal *et al.,* 1995). Whereas a marked potentiation was reported against *L.donovani in vitro*, a less-pronounced, additive effect of the antimonials drug was noted in mice (Neal *et al.,* 1995). Another study specifically focused on interactions in efficacy between miltefosine and sodium stibogluconate, amphotericin B, paromomycin, and sitamaquine (Seifert and Croft, 2006). *In vivo*, the highest enhancement of miltefosine activity was seen with amphotericin B, which preceded paromomycin. No activity enhancement was seen with miltefosine combined with sodium stibogluconate. Whereas the combination of miltefosine and amphotericin B could theoretically have some advantages over the other combinations, its clinical relevance remains unknown. More recent findings have also shown a synergistic interaction between amphotericin B and paromomycin (Seifert *et al.,* 2006).

3.3 Clinical data on combination therapy

The combination of pentavalent antimonials and paromomycin was the first regimen to be studied in India, at a time when clinical failure with pentavalent antimonials was increasingly being reported (Olliaro *et al.,* 2005). Overall, these studies showed that 21-day regimen of paromomycin as monotherapy or combined with pentavalent antimonials were efficacious for visceral leishmaniasis. Subsequently, promising data became available on (shortened) monotherapy regimens. A phase 2 study showed that even with liposomal amphotericin B given as a single dose (5 mg/kg), a high proportion of patients could be cured (Sundar *et al.,*2001, 2003). Equally high proportions could be achieved with 14 day treatment of miltefosine (Sundar *et al.,* 2000b). These observations provided the rationale for a phase 2, non-comparative randomised trial in India, which assessed different combinations of a single dose of liposomal amphotericin B followed by miltefosine for 7–14 days (Sundar *et al.,*

2008). All combinations were highly efficacious (≥95% of patients cured) and well tolerated, irrespective of the duration of miltefosine treatment.

DND*i* has conducted several VL clinical studies in three continents (Asia, East Africa and Latin America). The objective of the Asia clinical project was to identify a safe and efficacious short course combination therapy using the three drugs registered in India; AmBisome, Paromomycin and Miltefosine. The goal was to have improved treatment options that can be implemented by national control programmes in India, Nepal and Bangladesh. Together with its partners from India, Nepal and Bangladesh, DND*i* has worked to find VL combination treatments, which are efficacious in Asia. Recently completed studies in India show a high efficacy of a single dose of AmBisome alone (95% cure rate) and combinations treatments (AmBisome and Paromomycin, AmBisome and Miltefosine, Paromomycin and Miltefosine), all for the treatment of VL in India. The combination study was designed to provide data for authorities in India, Nepal and Bangladesh to make informed recommendations for combination treatments, which can be used in the VL elimination programmes in the region. This study involving 634 patients was completed in 2010. All three combination treatments were highly efficacious (≥97.5% cure rate), and none was inferior to standard treatment Amphotericin-B. This data therefore indicate that these combination treatment offer shorter, safer and cheaper treatment options than the current standard monotherapy treatment available in the region. A two step phase III trial (first in hospital settings followed by treatment in primary health care centres) using the same combination has now been initiated in Bangladesh. DND*i* is now actively collaborating with the national control programmes and health authorities, and with other international partners including WHO-TDR and iOWH, to facilitate introduction of new treatments for VL in South Asia (DND*i*'s meeting, 2010). In Africa, combination therapy of sodium stibogluconate and paromomycin was studied in the late 1980s, (Chunge *et al.*, 1990) and was subsequently used

by Médecins Sans Frontières, who needed a shorter treatment regimen when faced with large numbers of patients during an epidemic in Sudan (Davidson *et al.,* 2009; Melaku *et al.,* 2007; Serman *et al.,* 1993, 1996). Retrospective cohort data from more than 4000 patients showed that, relative to monotherapy with pentavalent antimonials (sodium stibogluconate), combination therapy was associated with clearly reduced mortality and fewer complications during treatment. This experience formed the basis for the leishmaniasis in east Africa platform (LEAP) 0104 trial, which was started in 2004 (final results are expected in early 2010) (Chunge *et al.,* 1990; Serman *et al.,* 1993; Melaku *et al.,* 1996, 2007; Wakabi, 2007; Davidson *et al.,* 2009; Anon, 2010a). This phase III trial, which was done in Sudan, Ethiopia, Kenya, and Uganda, initially compared two monotherapy regimens-sodium stibogluconate (20 mg/kg for 30 days) and paromomycin sulphate (15 mg/kg for 21 days)-with the combination of both drugs at the same dose for 17 days (table 2) (Anon, 2010a). In 2006, because of unexpectedly low efficacy with paromomycin monotherapy, the protocol was amended, and the dose of paromomycin was increased to 20 mg/kg in the second monotherapy group (Mudawi, 2009). Whether the low efficacy related to drug resistance, differences in susceptibility or pharmacokinetics is currently being investigated. No large trials on other combination regimens have been done in Africa. Studies on miltefosine and amphotericin B as monotherapy are limited due to involvement of children into the study (Berman *et al.,* 1998; Mueller *et al.,* 2006, 2008; Ritmeijer *et al.,* 2006).

3.4 Cost-effectiveness

Combination therapies have the potential to reduce the cost to the public health system and patients by reducing the duration of treatment. This not only lowers the burden to the health system but also reduces the economic inactivity of patients. Preliminary findings on the cost-effectiveness of combination therapy in India, Nepal, and Bangladesh, showed them to be a

viable alternative to monotherapy, with liposomal amphotericin B and paromomycin the best combination economically (Meheus *et al.*, 2009).

3.5 Prevention of drug resistance

Although pentavalent antimonials have been successfully used throughout the world for decades, poor treatment response (mainly due to initial treatment failure) has increasingly been reported since the 1980s from Bihar, India, with geographical and temporal clustering in several hyper endemic districts (Peters, 1981, Sundar, 2001). Although treatment outcomes could initially be improved with higher total doses, the improvement was only temporary (Thakur *et al.*, 1984, 1988, 1991). In subsequent reports, therapy failed in up to 60% of patients that were newly diagnosed (Sundar *et al.*, 1995, 1997, 2000). At the same time, misuse of the drugs was reported (Sundar *et al.*, 1994). Increased treatment failure has also been reported in Nepal, in districts that neighbour Bihar (Rijal *et al.*, 2003, 2009). Although treatment failure can have several causes, including factors related to drug, host, and parasite, substantial evidence exists that acquired drug resistance is a key issue. Reduced drug sensitivity has been reported with *L. donovani* strains from nonresponsive cases *in vitro* (Lira *et al.*, 1999; Dube *et al.*, 2005; Laurent *et al.*, 2007). Reduced susceptibility to pentavalent antimonials has also been reported with *L. infantum* in both human beings and animals (Faraut-Gambarelli *et al.*, 1997; Carrio *et al.*, 2001, 2002). In these studies, post-treatment isolates had reduced sensitivity compared with pre-treatment isolates, supporting the notion of acquired drug resistance. However, more recent studies have reported less clear associations of *in-vitro* susceptibility and clinical outcomes, underscoring the need of improved and standardised methods (Rijal *et al.*, 2009). The limited understanding of the mechanism of resistance towards pentavalent antimonials, and the shortcomings of drug sensitivity assays, make it difficult to predict the risk of acquired resistance in other regions or drugs and to assess the need for combination therapy to help prevent resistance. However,

on the basis of the evidence, acquired drug resistance should be thought to be a potentially serious threat to visceral-leishmaniasis control, and comprehensive strategies should be developed, including the use of combination therapy (Bryceson, 2001; Sundar, 2001; Croft *et al.,* 2006; den-Boer *et al.,* 2009).

3.6 Combination therapy using immunomodulator

The efficacy of treatment of Leishmania is compromised due to suppression of immune function during the course of infection (Bogdon, 2008). It is usually associated with a depression of Th1 cells and preferential expansion of Th2 cells and accordingly, skewing of T helper cells towards a Th1 response is considered as a promising therapeutic strategy. There are several studies using endogenous biologicals, microbial derivatives or synthetic compounds in both animals and humans. Amongst them most frequently used immunomodulators were BCG (Bacille Calmette–Guèrin), MDP (muramyl dipeptide), trehalose mycolate, glucan, tuftsin, and protein-A which have a direct effect on macrophages (Sundar *et al.,* 2007). Previous reports showed that biological immunomodulators such as IFN-γ (interferon- gamma) (Murray *et al.,* 1988) and immiquimod (Buates and Matlashewski, 1999; Arevalo *et al.,* 2001) have enhanced the activity of antimonials in the treatment of VL. Immunomodulator, imiquimod in combination with paromomycin effectively treated cutaneous leishmaniasis caused by *L. major* (El-On J *et al.,* 2007). Croft *et al.* (2000) evaluated antileishmanial efficacy of "tucaresol" against *L. donovani* in BALB/c mice and found it to be moderately effective (Smith *et al.,* 2000). To evaluate further the usefulness of immunomodulators in parasitic diseases, Guru *et al.* (1989) and Agrawal *et al.* (2002) tested tuftsin bearing liposomes as a vehicle to deliver SSG and amphotericin-B respectively in *L. donovani* infected hamsters. Results of these studies demonstrated that tuftsin bearing liposomes besides delivering the drug to the target cells could also enhance the nonspecific resistance against leishmanial infection (Guru *et al.,* 1989; Agrawal *et al.,* 2002). Adjunct

therapy of muramyl peptide with stibanate against VL in hamsters was also found quite effective (Puri *et al.,* 2005). Antileishmanial efficacy of miltefosine was also found to be enhanced when given in combination with a potent immunomodulator, picroliv (Gupta *et al.,* 2005). Solgi *et al.* (2006) showed effective application of immunomodulator "thalidomide" in combination with glucantime for treatment of *L. major* infection in BALB/c mice (Solgi *et al.,* 2006). A bacterial polysaccharide "Z-100" in combination with meglumine antimoniate controlled both the parasite load and the footpad swelling caused by *L. amazonensis* (Barroso *et al.,* 2007). Interferon- γ is one of the principal activators of macrophages. Clinical trials with IFN- γ alone and/or in conjunction with Sb^v were undertaken. With Sb^v it was reported to be useful in treating severe or Sb^v refractory VL in Brazil, however, in India in a large randomized study comparing Sb^v alone with Sb^v plus IFN-γ for 15 or 30 days had disappointing results as the final cure rate with Sb^v plus IFN-γ was 42 and 49 per cent, respectively (Sundar & Chatterjee, 2006). Based on these findings, we have explored the effect of an immunomodulator and hepatoprotective agent picroliv in combination with standard antileishmanials paromomycin and miltefosine in *L.donovani*/hamster model. Along with this we have also studied the effect of another immunomodulator CpG ODN in combination with miltefosine in mouse and hamster models of *L.donovani*.

3.7 Immunomodulators used for combination therapy in present work

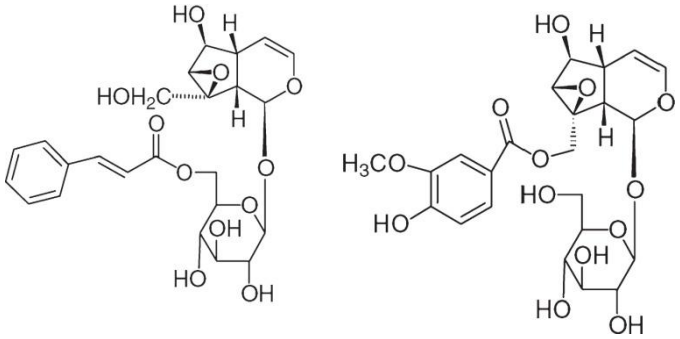
3.7.1 Picroliv (*Picrorhiza kurroa*)

Picrorhiza kurroa Royle ex. Benth (Family Scrophulariaceae), a small perennial herb, is found in the Himalayas from Kashmir to Sikkim at an altitude of 2700-4500 m. The plant is used as a bitter tonic in traditional medicine and hence commonly known as 'kutki'. The rhizomatous part of the plant and the root is used in dyspepsia, fever and also in the diseases of liver and spleen including jaundice. **Arogyavardhini**, a herbo-mineral preparation containing *P. kurroa* as the major ingredient has been tried in patients with viral hepatitis.

'Picroliv' isolated from this plant is an active hepatoprotective agent (Luper, 1998; Satyavati *et al.,* 1987). It is useful as a laxative, liver-stimulant, improving lactation, appetite stimulant, and febrifuge. It also exhibits anti-inflammatory (Singh *et al.,* 1993) antidiabetic and immunoregulatory functions (Langer *et al.,* 1981). Picroliv has also been found to possess active hepatoprotective activity against different hepatotoxins (Saraswat *et al.,* 1999).

3.7.1.1 Chemistry

The most important active constituents of *P. kurroa* are the iridoid glycoside, picroside I, II, III and kutkoside, collectively known as 'kutkin' (Luper, 1998). A stable mixture of picroside I and kutkoside at a ratio of 1:1.5 is named as 'picroliv'. This constitutes at least 60% of the total constituents previously stated as 'kutkin'. The remaining 40% is a mixture of iridoid glycosides as well as cucurbitacin glycosides and some unidentified substances.



Picroside-I **Kutkoside**

3.7.1.2 Pharmacokinetics

The active constituents from *P. kurroa* (picroliv and kutkin) are poorly soluble in water but soluble in ethanol. The drug cannot be administered in the form of tea as it is insoluble in water and in the form of tincture as it is not palatable. Therefore, the drug is administered as an encapsulated standardized extract (4% kutkin). The usual adult dose is 400–1500 mg/day, although daily dose as high as 3.5 g/day has been recommended for fevers. After intravenous administration, picroside I was mainly distributed in the central compartment and was rapidly eliminated from the plasma. There was no significant effect on the main pharmacokinetic data

when different doses of picroside I were administered and the kinetics seemed to be linear in nature (2.5–15 mg/kg) (Jing Lv *et al.*, 2007). The fifty percent lethal dose (LD) of kutkin is greater than 2600 mg/kg in rats (Luper, 1998). Picroliv showed an LD50 value of 2026.9 mg/kg in mice when administered intraperitoneally. No mortality was found up to 2.5 g/kg po dose in mice. By comparison, the maximum dose achievable with *picrorhiza* root is about 3-6 mg/kg (Luper, 1998; Negi *et al.*, 2007).

3.7.1.3 Mechanism of action

Picroliv prevented paracetamol-induced lowering of low density lipo-protein (LDL) receptor cell surface expression and increased conjugated dienes in hepatocytes. In rats infected with *Plasmodium berghei*, picroliv restored depleted glutathione levels, thereby enhancing detoxification and antioxidation. Thus, picroliv maintains a normal oxidation-reduction balance and glutathione metabolism and reduces the increased levels of lipid peroxidation products in the liver (Negi *et al.*, 2007).

Picroliv showed liver regenerative activity in rats, possibly by stimulating nucleic acid and protein synthesis (Singh *et al.*, 1992). Its hepatoprotective effect appears to result from a combination of membrane stabilizing, hypolipidemic and antioxidant properties. These properties may also be responsible for the effects on the immune system (Negi *et al.*, 2007).

3.7.1.4 Pharmacological actions

Most of the research work on *P. kurroa* has focused on its hepatoprotective, anticholestatic, antioxidant, anti-inflammatory and immune-modulating activities.

3.7.1.5 Hepatoprotective actions

The active ingredient, picroliv, has been shown to produce hepatoprotective activity against thiocetamide, galactosamine, rifampicin and cadmium-induced liver toxicity in cell culture and in experimental animals (Dwivedi *et al.*, 1991, 1992; Saraswat *et al.*, 1997; Yadav and Khandelwal, 2006). At doses of 6 and 12 mg/kg, picroliv provided hepatoprotection against

carbon tetrachloride induced alterations in biochemical parameters, viz. alanine transaminase, aspartate transaminase, bilirubin, protein, cholesterol triglycerides and lipoprotein X (Dwivedi *et al.,* 1990).

3.7.1.6 Choleretic and anticholestatic actions

Picroliv showed a dose-dependent (1.5–12 mg/kg x 7days) choleretic activity in conscious rats and anaesthetized guinea-pigs. It also possessed a marked anticholestatic effect against paracetamol and ethinylestradiol induced cholestasis. It antagonized the changes in bile volume as well as in bile salts and bile acids. Picroliv was found to be a more potent choleretic and anticholestatic agent than flavonolignan and silymarin (Shukla *et al.,* 1991). Picroliv induces the bile salt-dependent fraction, thereby increasing the synthesis of bile salts and bile acids, and enhancing conjugation with proteins (Saraswat *et al.,* 1997).

3.7.1.7 Antioxidant actions

The hepatoprotective activity of picroliv is mainly attributed to its antioxidant and stabilizing actions on the cell membranes of hepatocytes. Picroliv acts as an oxygen free radical scavenger that limits lipid peroxidation involved in membrane damage elicited by hepato-toxins. In aflatoxin B1-induced lipid peroxidation models in rats, picroliv produced protective effects comparable with that of the standard drug silymarin in normalizing elevated lipid peroxide levels as well as antioxidant enzymes (Rastogi *et al.,* 2001). Picroliv has been reported to improve the activity of the hepatic cytochrome P-450 enzyme system and protected liver from CCl4-induced hepatotoxicity. It also prevented the depletion of reduced glutathione, which is needed for the GST for detoxification reaction and for raising the levels of lipid peroxides in the liver (Rastogi *et al.,* 1997). Picroliv showed hepatoprotective effects in chronic alcohol-intoxicated rats. The levels of alcohol-metabolizing enzymes have been found to be increased in hepatocytes after picroliv treatment, suggesting the inhibitory effect

on the accumulation of acetaldehyde. Moreover, it restored glycogen, protein and lipid levels in the liver tissue and the redox potential in mitochondria (Saraswat *et al.,* 1999).

3.7.1.8 Antiviral actions

Picroliv was found to act against hepatitis B virus. It has anti-HBsAg like activity and inhibited purified HBV antigens prepared from healthy HBsAg carriers (Mehrotra *et al.,* 1990). Anti-inflammatory action *P. kurroa* extracts have an inhibitory effect on proinflammatory cells such as neutrophils, macrophages and mast cells (Pandey and Das, 1989). Apocynin, a catechol fraction from *P. kurroa,* has been found to exhibit powerful anti inflammatory actions on a variety of inflammatory models. It was found to inhibit neutrophil oxidative burst *in vitro* without affecting beneficial activities such as chemotaxis, phagocytosis and intracellular killing of bacteria (Luper, 1998).

3.7.1.9 Immunomodulatory action

The immunostimulant activity of picroliv was demonstrated in mice, which were immunized with sheep blood cells. Picroliv enhanced the non-specific immune response characterized by an increase in macrophage migration index (MMI), [14C]-glucosamine uptake, phagocytosis of [14C]-leucine-labelled *E.coli,* chemiluminescence of peritoneal macrophages and higher uptake of [3H]-thymidine in the lymphocytes of treated mice (Puri *et al.,* 1992). Picroliv inhibited hepatocarcinogenesis induced by N-nitrosodiethylamine in rats (Rajeshkumar and Kuttan 2000).

3.7.1.10 Clinical trials

In a randomized double-blind placebo-controlled trial, 15 patients with acute viral hepatitis were given *P. kurroa* root powder (375 mg, three times a day for 2 weeks). *P. kurroa* produced faster relief from anorexia, nausea and malaise when compared with placebo. There was no serious adverse effect with *P. kurroa* treatment (Vaidya *et al.,* 1996). Picroliv

successfully completed phase I and phase II clinical trials and is presently under Phase III trial. It is expected to be in the market very soon for human use.

3.7.1.11 Safety profile

Long-term toxicity studies conducted on histopathological parameters in rats showed picroliv was non-toxic. A similar experiment conducted on adult rhesus monkeys showed no abnormality in food intake, daily activities, body weight, blood biochemistry and haematology. Its safety has also been demonstrated in human beings (Negi *et al.,* 2007).

3.7.1.12 Future prospects

Traditionally, *P. kurroa* is used as a liver tonic in Aarogyavardhini Rasa, which has proved its safety and efficacy. *P. kurroa* root powder and picroliv as active constituents may be of use in viral hepatitis. The antioxidant, anti-inflammatory, antiviral, immunomodulatory, liver regenerative, anti-lipid peroxidative property as well as the ability to prevent free radical damage may prove to be very useful in hepatotoxicity induced by viral agents, toxic drugs and plant poisons. The high safety profile may be an added advantage. The clinical trials of picroliv may provide new insight into its safety, efficacy and tolerability. Further, they may be combined with other hepatoprotective and/or antiviral agents.

3.7.2 CpG oligodeoxynucleotides (ODN)

CpG ODNs are a recently described class of pharmacotherapeutic agents that are characterized by the presence of an unmethylated CG dinucleotide in specific base sequence contexts (CpG motif). These CpG motifs are present in bacterial DNA to which they confer immunostimulatory properties but are not seen in eukaryotic DNA, in which CG dinucleotides are suppressed and, when present, usually methylated (Krieg *et al.,* 1995). These immunostimulatory properties include induction of a Th1-type response with prominent release of IFN-γ, IL-12, and IL-18. CpG ODNs possess immunomodulatory properties similar to bacterial DNA (Kline & Kreig, 2001).

3.7.2.1 History

The first reported systematic attempt to use an immunostimulatory therapy for a non bacterial disease took place in the 1890s when Dr. William Coley, a New York surgeon, performed a series of studies evaluating the anti-tumor activity of bacteria. In his initial studies, he injected live *streptococci* directly into the tumor masses of his patients. This resulted in tumor regression in his first patient that lasted for 7 years. However, dangers of infection were high, with the first patient almost dying of erysipelas. For the next two decades, Coley explored use of heat-killed gram-positive and gram negative bacteria as immunotherapeutic agents for cancer (Coley, 1893 and 1894).This preparation, known as **"Coley's Toxin,"** resulted in tumor regression in some patients, although the response rate was less than that seen with live organisms. In subsequent decades, much of the antitumor activity of Coley's toxin was attributed to endotoxin (Wiemann and Starnes, 1994). Additional bacterial components, such as bacterial DNA, may well have played a role in the observed responses. It was almost 100 years between Coley's studies and the recognition that bacterial DNA itself can stimulate the immune system. Shimada, Yamamoto and colleagues demonstrated that bacterial DNA could enhance natural killer (NK) cell activity (Shimada *et al.,* 1986; Yamamoto *et al.,* 1992) and Messina, Pisetsky, and colleagues found that such DNA was also capable of inducing B cell activation (Messina *et al.,* 1991). In the mid-1990s, Krieg and colleagues gained additional insight into the moieties in bacterial DNA that are responsible for the immunostimulatory effects of bacterial DNA. After making and testing hundreds of ODN, they determined that the immunostimulatory effects of these "control" ODN were dependent on an unmethylated CpG dinucleotide in a particular sequence context (Krieg *et al.,* 1995). Since then, synthetic CpG ODN has been the focus of intense research due to the Type I pro-inflammatory response they elicit and their successful use as vaccine adjuvant. Klinman *et al.,* found these ODN not only induced B cell activation, but also induced production of a

wide variety of cytokines, indicating a more complex pattern of immune activation (Klinman *et al.,* 1996). Following these facts it was said that bacterial DNA serve as a “danger signal”. The unmethylated CpG motifs enable the mammalian immune system to distinguish microbial DNA from self DNA. Sequence differences between bacterial DNA and vertebrate DNA appear to make this possible. CpG dinucleotides are present at the expected frequency in bacterial DNA (1 in every 16 dinucleotides) while mammals have CG suppression, with CG dinucleotides being found at approximately one-fourth the expected frequency (Bird, 1987). Furthermore, the majority of cytosines present in CG dinucleotides are methylated in mammals. Although bacteria can methylate select bases, there is no methylation specificity for CG dinucleotides. Thus, unmethylated CG dinucleotides are much more common in bacteria than in mammals and other vertebrates.

3.7.2.2 Structural Features

Synthetic CpG ODN differ from microbial DNA in that they have a partially or completely phosphorothioated (PS) backbone instead of the typical phosphodiester backbone and a poly G tail at the 3' end, 5' end, or both. PS modification protects the ODN from being degraded by nucleases such as DNase in the body and poly G tail enhances cellular uptake (Dalpke *et al.,* 2002). The poly G tails form intermolecular tetrads that result in high molecular weight aggregates. These aggregates are responsible for the increased activity the poly G sequence impart; not the sequence itself (Wu *et al.,* 2004). Numerous sequences have been shown to stimulate TLR9 with variations in the number and location of CpG dimers, as well as the precise base sequences flanking the CpG dimers. This lead to the creation of five unofficial classes or categories of CpG ODN based on their sequence, secondary structures, and effect on human peripheral blood mononuclear cells (PBMCs). The five classes are Class A (Type D), Class B (Type K), Class C, Class P, and Class S (Vollmer and Krieg, 2009). It is important to note that during the discovery process, the "Classes" were not defined until

much later when it became evident that ODN with certain characteristics elicited specific responses. Because of this, most ODN referred to in the literature use numbers (i.e. ODN 1826, ODN 2006, ODN 2007, ODN 2216, ODN D35, ODN K3 etc.).

Class A

One of the first Class A ODN, ODN 2216, was described by Krug *et al.* (2001). These are characterized by a phosphodiester central CpG-containing palindromic motif and a phosphorothioate 3' poly-G string.CpG ODN of the A class (CpG-A ODN) are very strong inducers of alpha interferon (IFN-á) by plasmacytoid dendritic cells (PDC) and are especially potent NK cell activators. Class A ODN typically contain 7 to 10 PS modified bases at one or both ends that resist degradation by nucleases and increase the longevity of the ODN.

Structural features defining Class A ODN

- The presence of a poly G sequence at the 5' end, the 3'end or both
- An internal palindrome sequence
- GC dinucleotides contained within the internal palindrome
- A partially PS modified backbone

Class B

Kreig *et al.,* was first to describe Class B ODN in 1995 (Krieg *et al.,* 1995). Type B CpG ODNs contain a full phosphorothioate backbone with one or more CpG dinucleotides. These are weaker inducers of IFN- á, but potent activators of B cells and monocyte maturation.

Structural features defining Class B ODN

- One or more 6mer CpG motif 5'-Pu Py C G Py Pu-3'
- A fully phosphothiorated (PS modified) backbone
- Generally 18 to 28 nucleotides in length

The strongest ODN in this class have three 6mer sequences (Hartmann *et al.*, 2000). B-ODN has been studied extensively as therapeutic agents because of their ability to induce a strong humoral immune response, making them ideal as a vaccine adjuvant.

Class C

CpG-C ODN has the combined features of CpG-A and CpG-B ODN. They contain a complete phosphorothioate backbone and a CpG-containing palindromic motif. They are strong inducers of PDC IFN- á /βproduction and strong B-cell activators. All classes of CpG ODN promote T helper 1 (Th1) responses to co-administered antigens through the induction of cytokines in activated dendritic cells.

3.7.2.3 Application of CpG ODN in Leishmania therapy

Initial findings (Zimmermann *et al.,* 1998) against *L. major* infection imply an important role of bacterial DNA and CpG-ODN in the instruction of adaptive immune responses. They also point to the therapeutic potential of CpG-ODN in redirecting curative Th1 responses in Th2 driven disorders. Results from the study of Stacey and Blackwell (1999) suggested that with an appropriate antigen, CpG ODN provide a stable, cost-effective adjuvant for use in vaccination against leishmaniasis and concluded that immunostimulatory DNA sequences exert systemic effects via IL-12 and IFN-γ-dependent mechanisms and hold considerable promise as both vaccine adjuvant and potential therapeutic agent in the prevention and treatment of leishmaniasis. Verthelyi *et al.* (2003) demonstrated that CpG ODN treatment of *Rhesus macaques* significantly reduced the severity of the lesions caused by a challenge with Leishmania. Datta *et al.* (2003) showed that liposomal CpG ODN inhibit amastigote multiplication in macrophages and increase the levels of IFN- γ , IL-12 (involved in upregulation of Th1 response) in spleen cells of Balb/c mice infected with *L. donovani* AG83 promastigotes with reduction in levels of IL-4. A Study by Jose *et al.* (2004) showed that mice vaccinated with a single dose of *L. major* Ag-pulsed BMDC stimulated by prior *in vitro*

exposure to CpG-containing oligodeoxynucleotides (ODN) were completely protected, had a dramatic reduction in parasite burden, and developed an Ag-specific Th1 response. Tewary *et al.* (2004a) investigated the effect of ODN containing immunostimulatory CG motifs as adjuvant with soluble antigen (SA) from *L. donovani* in BALB/c mice. CpG-ODN alone resulted in partial protection against challenge with *L. donovani* metacyclic promastigotes. SA and CpG-ODN showed enhanced reduction in parasite load (~60%) when compared to SA (~40%). Leishmania-specific Th1 cytokine response was induced by co-administering CpG-ODN and SA as they together promoted production of IFN-γ, IL-12 and immunoglobulin isotype IgG2a. Tewary *et al.* (2004b) vaccinated CpG ODN in combination of rORFF in BALB/c mice followed by a challenge with *L. donovani* metacyclic promastigotes. Administration of CpG-ODN alone resulted in partial protection against challenge with *L. donovani* in BALB/c mice. Combination of rORFF and CpG-ODN showed enhanced reduction in parasite load (84%) when compared to rORFF (56%) vaccinated mice. Results further demonstrate that CpG-ODN alone or in combination with rORFF resulted in a dose dependent increase of nitric oxide production in activated macrophages. Flynn *et al.* (2005) administered D/A ODN to macaques following an intradermal infection with *L. major* and observed reduced severity of the lesions. This findings supports the development of clinical studies to assess the use of CpG ODN types D/A as immunoprotective and therapeutic agents. Iborra *et al.* (2005) challenged two models of murine cutaneous leishmaniasis: (i) subcutaneous inoculation of *L. major* parasites in susceptible BALB/c mice (a model widely used for vaccination analysis) and (ii) the intradermal inoculation of a low infective dose in resistant C57BL/6 mice (a model that more accurately reproduces the *L. major* infection in natural reservoirs and in human hosts). C57BL/6 mice vaccinated with LiP0-DNA or rLiP0 protein plus CpG oligodeoxynucleotides (ODN) were protected against the development of dermal pathology and showed a reduction in the parasite load. This

protection was associated with production of gamma interferon (IFN-γ) in the dermal site. Secondly immunization with rLiP0 plus CpG ODN is able to induce only partial protection in BALB/c mice. Rafati *et al.* (2005) vaccinated by prime/boost with DNA/recombinant CPs (in combination with CpG ODN and Montanide 720) and challenged dogs with *L. infantum* and were followed for 12 months. Ten dogs vaccinated remained free of infection in their bone morrow. Analysis of cytokine mRNA level suggested that vaccinated dogs had elevated IFN-γ mRNA in peripheral blood mononuclear cells (PBMC), whereas there was a consistent increase in the level of IL-10 in the control groups and some vaccinated dogs. Badiee *et al.* (2008) co-encapsulated CpG ODN with Recombinant *Leishmania major* Stress-Inducible Protein 1 in Liposome and observed enhancement in immune response and protection against Leishmaniasis in Immunized BALB/c mice. Iborra *et al.* (2008) vaccinated CpG ODN with *Leishmania major* ribosomal proteins in Balb/c mice followed by a challenge with *L. major* and observed reduction in parasitic load and enhancement in TH1 response with an increased level of IFN-γ and IL12 cytokines.

Based on the above findings, the effect of combination of class B CpG oligodeoxynucleotides (ODN 1826 and 2006) and miltefosine at sub-curative dose for the treatment of experimental visceral leishmaniasis in mouse and hamster models was studied. The hamster model is known to closely mimic the condition of human visceral leishmaniasis better than any other animal model. The study involves use of free as well as liposomal CpG ODN (1826 and 2006) in the combination with miltefosine. Detailed study of immunological parameters namely Th1/Th2 cytokines, production of ROS, RNS, H_2O_2 and phagocytosis was done to establish the effect CpG ODN 1826 on the immune functions of the host (mouse and hamster model). The results were compared with untreated animals which served as control.

CHAPTER-4

MATERIALS AND METHODS

It is broadly divided into *in vitro* and *in vivo* system:

4.1 *In vitro* system

4.1.1. Parasite

The parasite, *Leishmania donovani* (strain MHOM/IN/80/Dd8), originally isolated from a human patient from Bihar (India) in 1979, was subsequently obtained through the kind courtesy of Prof. P.C.C. Garnham of Imperial College, London, in 1981. Since then it was regularly maintained in this laboratory (Gupta *et al.*, 2005).

Medium used

- **M-199 medium**

Powdered 199 medium (Sigma, USA) and gentamycin along with the below mentioned ingredients were dissolved in 500ml TDW and stirred over a magnetic stirrer for 30min to obtain a clear solution. The rest of volume of the medium was reconstituted with TDW and pH was adjusted to 7.2 ± 0.2 with the help of 1N: HCl/ or 1N: NaOH. The solution was then filtered through filter assembly of 0.2 μm pore size and tested for sterility by incubating few ml of medium at 37ºC for 24h. Sterile medium was stored at 4ºC until use. To make complete M-199 medium, 10% Foetal Bovine Serum (FBS from Hyclone) was added prior to use and filtered again.

Composition:

- ✓ Powdered medium - 14.6 g
- ✓ Sodium bicarbonate ($NaHCO_3$)- 2.2 g
- ✓ Gentamycin – 4mg
- ✓ TDW - 900mL

Maintenance of the parasite

Promastigotes of *Leishmania*, though a form which exist in sand fly can easily be grown *in vitro* using simple biphasic or monophasic media. Leishmania parasites were maintained in routine culture in M-199 medium (monophasic) supplemented with 10% FBS at 24ºC ± lºC. Subcultures were carried out after 3-5 days. However, the period of subculture may vary depending upon the inoculum seeded at the time of subculture and also the status of each culture. During every subculture, smears of sample are prepared on slide aseptically and observed under microscope.

4.1.2. Cell line

Mouse macrophage cell line (J-774A.1) was obtained from the Tissue Culture Laboratory, CDRI, Lucknow.

Medium used:

- **RPMI-1640 (Rosewell Park Memorial Institute- 1640)**

Powdered RPMI medium (Sigma, USA) along with the below mentioned ingredients were dissolved in 500ml TDW and stirred over a magnetic stirrer for 30min to obtain a clear solution. The rest of volume of the medium was reconstituted with TDW and pH was adjusted to 7.2 ± 0.2 with the help of 1N: HCl/ or 1N: NaOH. The solution was then filtered through filter assembly of 0.2 µm pore size and tested for sterility by incubating few ml of medium at 37ºC for 24h. Sterile medium was stored at 4ºC until use. To make complete RPMI medium, 10% FBS was added prior to use and filtered again.

Composition:

- ✓ Powdered medium 13.3 g
- ✓ Sodium bicarbonate (NaHCO3) - 2.0g
- ✓ Glucose - 2.0g

- ✓ HEPES buffer - 5.94g
- ✓ Gentamycin – 4mg
- ✓ Triple distilled water (TDW) - 900mL

Maintenance of the cell line

J-774A.1 cells are being continuously maintained using RPMI-1640 medium with 10% FBS in incubator at 37^{o}C temperature and 5% CO_2 supply conditions. The cell lines were subcultured on day at which 100% confluence reached.

4.1.3. Methods used in *in vitro* system

- **Anti amastigote activity**

On day 0 resident peritoneal macrophages were induced by injecting, intraperitoneally 2ml of 2% starch solution into each mouse 24h prior to cell extraction. On day 1, peritoneal macrophages from BALB/c mice were harvested by lavage in sterile conditions. The abdominal surface of mice was liberally sprayed with 70% ethanol and then 4-5 ml of ice cold RPMI -1640 medium containing 4 µg/mL gentamicin and 5% EDTA was introduced inside the abdominal cavity by 10 ml disposable syringe. Peritoneal fluid was collected and centrifuged in cold centrifuge at 1000 rpm for 10 min. The cells were washed and resuspended in complete medium (RPMI + 10% FBS). Cells were counted using Neubauer hemocytometer and adjusted with complete medium to get desired concentration needed for the experiment.

More than 95% macrophage cells found to be viable by 0.04% trypan blue dye exclusion method were used for various experiments. A population of 1x10^{6} cells per mL per 100µL/well was suspended in complete RPMI medium and layered in 16 well chamber slides (Nunc). Chamber slides were then incubated for 24h in 5% CO_2 incubator at 37^{o}C. On day 2 spleen of an infected hamster was aseptically extracted and washed with PBS and its weight

is noted. Dab smear of a small portion of spleen tissue was made which was fixed with 100% methanol and stained with 20% Geimsa stain for 45 minutes. Dab smear of spleen was counted microscopically to get total number of amastigotes per 500 cell nuclei. Small pieces of spleen were then transferred to a sterile glass homogenizer and pulped to get homogenous solution. The homogenate was transferred to a sterile 50ml centrifuge tube and topped with 45ml RPMI 1640.This was then centrifuged, in a cold centrifuge, at 800-1000rpm for 10 min to separate RBCs and small tissues. The supernatant was transferred to another sterile 50ml tube and centrifuged at 3100 rpm for 15 min. The supernatant was discarded and the resulting pellet was re-suspended in 1ml cold complete RPMI.

Total amastigote burden of the organ was calculated by the Stauber equation (Stauber *et al.*, 1958).

$$\textbf{Number of amastigotes in organ} = \frac{\textbf{No. of amastigotes}}{\textbf{No. of cell nuclei}} \times \textbf{weight of organ (mg)} \times 10^5$$

These amastigotes were added to each well of 16 well chamber slides in ratio of 1:7 and incubated for 24h in 5% CO_2 incubator at 37^oC. On day 3 drugs were prepared in complete RPMI medium and added in appropriate concentrations. Drug containing medium was changed on day 5. Finally slides were fixed with 100% methanol and stained with 20% Geimsa stain for 45minutes on day 6. The number of amastigotes per 500 cell nuclei was counted in each well and the parasitic burden was expressed in terms of the number of amastigote per 100 cell nuclei. Drug activity (percent inhibition) was determined by comparing amastigote count of treated and untreated wells by the general formula:

$$\textbf{Percent Inhibition} = \frac{\textbf{N-n}}{\textbf{N}} \times \textbf{100}$$

Where,

N is average number of amastigotes per 100 cell nuclei of untreated well and **n** is average number of amastigotes per 100 cell nuclei of treated well.

- **Apoptotic-Necrotic profiling to determine phosphatidylserine exposure in promastigotes**

To establish whether the cell death is via apoptosis or necrosis, apoptotic – necrotic profiling of untreated control and drug treated *L.donovani* promastigotes was carried out. Annexin-V-FITC apoptosis kit (Sigma) was used for this purpose as per manufacturer's instructions. Kit includes Annexin V-FITC (fluoroscein isothiocynate) which labels phosphatidylserine sites on cell membrane and represent apoptotic cell death. A cell impermeable nuclear stain PI (Propidium Iodide) was used to label the cellular DNA in necrotic cells where the cell membrane has been totally compromised. PI is a counter stain. Positioning of quadrants on Annexin V/ PI dot plots was performed and living cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+) were distinguished as described by Vermes *et al.* (1995). Promastigotes in log phase were centrifuged and suspended in complete M-199 medium. A population of 2million promastigotes per ml were seeded in 24 wells plate (1ml/well).These promastigotes were treated with individual as well as combination of drugs in different concentrations and then incubated for 72h at 24 ± 2^oC. For measuring apoptotic – necrotic profile, treated and untreated promastiogotes were washed with PBS by centrifugation (3100 rpm for 10 min.) followed by addition of 5 µL Annexin V- FITC and 10 µL PI to each tube. Samples were incubated for 30 minutes at room temperature. Fluorescence of cells immediately determined with Cell Quest FACS Caliber (Becton Dickinson). Cells were analyzed by flow cytometry with Ex: 488 nm and Em: 530 nm for Annexin V-FITC detection (FL1 channel) and for PI filter > 600 nm was used (FL2 channel). Electronic compensation was done to exclude overlapping of the two emission spectra.

- **Measurement of mitochondrial transmembrane potential drop in promastigotes.**

The dissipation of mitochondrial membrane potential is a characteristic of apoptosis. To assess the mitochondrial transmembrane electrochemical gradient (ΔΨm), a cell-permeable, cationic and lipophilic dye, JC-1, was used. This probe aggregates within mitochondria and fluoresce red at higher transmembrane potentials. However, at lower transmembrane potentials, JC-1 cannot accumulate within the mitochondria and instead remains in the cytosol as monomers, which fluoresce green. Therefore, the ratio of red to green fluorescence gives a measure of the transmembrane electrochemical gradient (Verma *et al.,* 2007, Sen *et al.,* 2007). The effect of different drug combinations on mitochondrial membrane potential of treated promastigotes was evaluated. Parasite in log phase were centrifuged and suspended in complete M-199 medium. A population of 2 million promastigotes per ml were seeded in 24 wells plate (1mL/well). These promastigotes were treated with individual as well as combination of drugs in different concentrations and then incubated for 72h at 24 ± 2^{o}C. After drug treatment, cells were washed in PBS by centrifugation at 3100 rpm for 10 min and incubated with JC-1(3mM) for 15 min at 20–25^{o}C and analysed by flow cytometry. Data acquisition was carried out using a FACS Calibur and analysed using CELLQUEST PRO software.

4.2. *In vivo* system

4.2.1. Animal

Healthy BALB/c mice (20 – 25g) and inbred hamsters (40-45g) of both sexes were used for the study. All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. Animals were bred in the National Animal Laboratory Centre, housed in CDRI, Lucknow. For experimental studies, animals were kept in plastic cages (38×27 ×13 cms) with husk as bedding. Four to six animals were assigned to each cage to avoid overcrowding. Throughout the study, the

animals were housed in climate-controlled (23 ± 2^{o}C; RH: 60%) and photoperiod-controlled (12hr light-dark cycles) animal quarters. They were fed standard rodent pellets diet supplemented with grain and had free access to drinking water.

4.2.2. Maintenance of the parasite *in vivo*

L. donovani parasites were maintained *in vivo* in BALB/c mice and hamsters through amastigotes to amastigotes by syringe passage or by inoculating stationary phase promastigotes. Inoculation of amastigotes (infection established within 15-20 days) is preferred over inoculation of promastigotes, which took relatively more time (2-3 months) for the infection to be established.

4.2.3. Methods used in *in vivo* system

- **Preparation of infective inoculum**

Hamsters with heavy infection (50-60 days old) were sacrificed, and their spleens removed aseptically in Locke's solution and cut into small pieces. The spleen pieces were pooled together and homogenized with the help of motor driven tissue homogenizer consisting of a glass tube and a Teflon pestle. The suspension was centrifuged at 800-1000 rpm at 4 ºC for 10 min to settle RBCs and small tissues. Supernatant was collected and recentrifuged at 3100 rpm for 15 min. The pellet containing amastigotes was finally resuspended in Locke's solution and the number of the parasites counted in Neubaur's heamocytometer. The inoculum was adjusted to contain 1×10^{7} amastigotes in 0.1 ml of suspension for passage in hamsters and 2×10^{7} amastigotes in 0.1 mL of suspension for passage in BALB/c mice. This infection is inoculated intracardically (Gupta *et al.*, 2005).

- **Assessment of established infection**

Infected hamsters at 18-22 days post-infection were subjected to general anesthesia of pentothal (Thiopentone sodium injection I.P., Abbott Laboratories, India, Ltd., Gujarat, India) (50 mg/kg, ip). The anesthetized hamsters (after 15-20 minutes) were kept on platform of

dissection tray and all four limbs were stretched and tucked with needles to rest hamster on its back. Hair from the left side of abdomen was shaved and an incision (less than 1 cm) made over the gastro splenic region to expose the spleen. One end of the spleen was pulled out with the help of forceps and 2-3 mm portion of the tissue was cut. The spleen was then inserted inside at its original position and layers of muscles and skin were separated sutured using curved needle (3/4", 22-25 gauge, half circle) and brained nylon stitch thread "00" number. Sutured portion was liberally sprinkled with Neosporin powder and finally sealed with thin cotton pad soaked in tincture benzoin. The complete operation was performed in sterile condition. The hamsters were administered neomycin sulphate (10 mg/kg) orally, one day prior to operation and at least on two consecutive days post operation to prevent any bacterial infection. In case of BALB/c mice, establishment of infection was checked in liver tissue by sacrificing the infected animal after 7 days post infection.

- **Assessment of parasitic burden**

A small portion, 5 mm size, of spleen/liver cut during biopsy/euthan was first blotted on filter paper (Whatman No.1) to remove excess of blood and then dab smears were prepared on glass microslides. The smear was quickly air dried, fixed in absolute methanol and stained with Giemsa dye (20% v/v in PBS, pH 7.2) for 45 min. These stained smears were observed under oil immersion (100X). The number of amastigotes was counted for 500 spleen cell nuclei and the parasitic burden was expressed in terms of the number of amastigote per 100 cell nuclei.

- **Biochemical analysis for measuring production of ROS, RNS and H_2O_2**

For fluorimetric detection of toxic oxygen (ROS & H_2O_2) and nitrogen (RNS) metabolites, dichlorofluorescein diacetate (DCFH) and diaminofluorescein-2-diacetate (DAF2DA) dyes were used respectively. 4α-Phorbol 12-myristate 13-acetate (PMA) was used as inducer for production of oxygen metabolites. Inhibitors used were pentoxifylline (PTX) which inhibit

NADP-oxidase which required for super oxide ion production, N-nitro-L-arginine methyl ester (L-NAME) which inhibit NO synthase essential for production of NO^- ion, and NaN_3 which inhibit catalase which is required for production of H_2O_2 (Warland *et al.*, 2003) Drop in fluorescence of inhibitor is an indicator of the concentration of its substrate in cell suspension. Fluorescence drop of inhibitor treated peritoneal exudate cells (PECs) was measured and compared with that of untreated PECs.

For PECs extraction abdominal surface of hamsters were cleaned with 70% ethanol and then 4-5 ml of ice cold RPMI -1640 medium containing 4 µg/mL gentamycin and 5% EDTA was introduced inside the abdominal cavity by 10 mL disposable syringe. Peritoneal fluid was collected and centrifuged at 800-1000 rpm for 10 min. The cells were washed, suspended, counted and adjusted with RPMI medium containing 10% FBS to get desired concentration needed for the experiment. More than 95% macrophage cells found to be viable by 0.04% trypan blue dye exclusion method were used for various experiments. Cells were layered at $1x10^6$ cells/mL per well in 24 well tissue culture plates and incubated at 37°C in 5% CO_2 for 24h. The non-adherent macrophages were removed by washing. Cells were incubated with inhibitors (L-NAME-10µM, PTX-10 µM and NaN_3-10 µM) for 1h at 37°C in 5% CO_2 followed by induction with PMA (20µM) and incubation of 1h. Finally 10µM of dyes (DCFH or DAF2DA) were added to each well and incubated for 30 min. Each step was followed by appropriate washings. Free radicals generated from peritoneal macrophages oxidized non fluorescent forms of dyes to fluorescent forms. Fluorescent signal from the dye was read on Cell Quest FACS Caliber (Becton Dickinson) with FL1 UV band pass filter (excitation at 488nm and emission at 510 to 513 ± 30nm).

- **Phagocytosis assay**

A flow-cytometry based method was used to study the phagocytic activity of macrophages (Sharma *et al.,* 2004). PECs of each experimental group extracted by the method described above and cells were seeded at $1x10^6$ cells/mL per well in 24 wells tissue culture plates and incubated at 37°C in 5% CO_2 for 24h. The non-adherent macrophages were removed by washing and incubated with 10 µM fluoroscein isothiocynate - labelled bacteria (1:10 ratio) for 30 min at 37°C, except control wells. After incubation, excess non phagocytized bacteria were removed by washing. The cells were collected in tubes and phagocytosis observed by FACS Caliber (Becton Dickinson) with FL1 UV band pass filter (excitation at 488nm and emission at 510 to 513 ± 30 nm. Results were represented as phagocytic index which was the ratio of mean OD of stimulated cells to mean OD of unstimulated cells.

$$\text{Phagocytic Index} = \frac{\text{Mean OD of Stimulated cells}}{\text{Mean OD of unstimulated cells}}$$

- **Lymphocyte proliferation assay**

Lymohocytes extracted from hamsters were used in this assay. Mesenteric lymph nodes from the treated and untreated animals were taken out in small Petri dishes containing plain RPMI medium and 40 µg/mL gentamycin. The nodes were washed with plain medium and were teased with a pair of fine needles and then sieved. This cell suspension was centrifuged at 1000 rpm/ 10min and counted. A cell population of 1×10^6 per mL, 200µL per well were seeded in 96 wells plate using RPMI medium supplemented with 4 µg/mL gentamicin and 10% heat-inactivated FBS. More than 95% viable cells, found with trypan blue dye exclusion test. Cells were stimulated with 25 µg / mL of SLA (Soluble Leishmania Antigen) and 5 µg / mL of Concanavalin A (standard stimulant) followed by incubation at 37°C in 5% CO_2 for

96h (Shrivastava *et al.*, 2003). One microcurie of methyl-tritiated thymidine (3H thymidine) was added to each well 18 h before harvesting. Cells were collected on to glass fibre filter paper discs and were harvested manually. The dried discs were placed in 8 mL of scintillation fluid (Buhler, 1962) and methyl-tritiated thymidine content measured in a LS 6500 multipurpose liquid scintillation counter (Beckman Coulter, USA). Data were expressed as count per minute (cpm) of the incorporation of 3H thymidine. The result was expressed as Stimulation Index which is described as the ratio of the mean count per min. of stimulated culture to that of unstimulated culture.

4.3. Cryopreservation of parasites and cell lines

For long-term preservation of the parasites and cell line obtained from culture were stored in liquid nitrogen using suitable cryoprotectant (Evans, 1989).

- **Freezing**

Prior to storage in liquid nitrogen, the parasite/cell suspension was gradually cooled at the rate of 1-2 ºC per min using "Mr Frosty" cryocontainer (Nalgene) in the presence of cryoprotectant (isopropanol) as described:

- Using aseptic conditions, log-phase promastigotes/cell culture (2×10^6 promastigotes/cells per ml) was transferred to cryo vials.
- Sterile cryoprotectant was added dropwise in the vial achieve the final concentration of glycerol (10-20%) or dimethyl sulphoxide (5-10%) and mixed thoroughly.
- The vials were placed in "Mr Frosty"box (pre cooled at -80 ºC for 24h) and kept at -80 ºC freezer for at least 24h. Then vials were transferred to liquid nitrogen container for storage.

Storage: The frozen samples known as stabilate were permanently stored at -80 ºC or shifted into liquid nitrogen (-196 ºC) until further use.

- **Thawing of stabilates**

Whenever required the parasites/cells were revived from the cryopreserved samples by the following method:

- The stabilates were thawed rapidly by plunging the sealed containers into warm water at about 37 ºC or by rolling them between the palms.
- The thawed sample was washed once with plain medium and transferred asceptically into complete culture medium.
- A sample of the thawed material was viewed under the microscope to check viability of the parasites/cell.
- The revived sample was checked daily for successful revival.

4.4. Statistical Analysis

Results are presented as mean ± S.D. of two experiments and analysis of data is carried out by Boneferroni's multiple comparison tests and Dunnett's multiple comparison tests. Differences with $P< 0.05$ were considered significant. Sub-curative dose was determined by probit analysis (Finney, 1971).

CHAPTER-5

IMMUNOMODULATORY EFFECT OF CpG OLIGODEOXY-NUCLEOTIDES (ODN) ON ANTILEISHMANIAL EFFICACY OF MILTEFOSINE

In the present work, combination of CpG oligodeoxynucleotides (ODN) and miltefosine (sub-curative dose) was studied for the treatment of experimental visceral leishmaniasis in rodent models. CpG ODNs are a class of pharmaco-therapeutic agents, characterized by the presence of an unmethylated CG dinucleotide in specific base sequence contexts (CpG motif). These CpG motifs are not seen in eukaryotic DNA, but are present in bacterial DNA to which they confer immuno-stimulatory properties (Kreig, *et al.,* 1995).The current study involves evaluation of antileishmanial efficacy of two Class B CpG ODNs namely ODN 1826 and 2006 in combination with miltefosine using mouse and hamster models. Previous studies confirm the applicability of class B CpG ODNs in the field of leishmaniasis, especially as immunomodulator and adjuvant with various immunogens (Walker *et al.,* 1999; Verthelyi and Klinmann, 2003; Datta *et al.,* 2003; Badiee *et al.,* 2008). In this study free as well as liposomal forms of CpG ODNs were used with sub-curative doses of miltefosine. This chapter includes detailed study of CpG ODN 1826 in mouse and hamster model supported with various biochemical and immunological parameters namely production of toxic oxygen and nitrogen metabolites (ROS, RNS, H_2O_2), phagocytosis assay, Th1/Th2 cytokines response and lymphocyte proliferation assay. The efficacy of CpG ODN 1826 was then compared with CpG ODN 2006 since this ODN has been extensively used in anti-tumor clinical trials (Jahrsdorfer and Weiner, 2008).

1. MATERIALS AND METHODS

1.1. Parasite

L. donovani (MHOM/IN/80/Dd8)

1.2. Animals

BALB/c mice (18-20 g) and inbred hamsters (40-45 g) of both sexes were used for the study.

1.3. CpG ODNs

Two fully phosphothioated type B CpG ODNs supplied by Trilink Biotechnologies Inc., San Diego, CA were used in study.

1. ODN 1826 is specific for mouse TLR9, having optimal mouse motif 5' GACGTT 3' and bear 18–24 bp length. Its sequence is 5' TCC ATG ACG TTC CTG ACG TT 3' and molecular weight is 6364 g/mol.
2. CpG ODN 2006 is specific for human TLR9, having optimal human motif 5' GTCGTT 3' (24 mer). Its sequence is 5'TCG TCG TTT TGT CGT TTT GTC GTT 3' and molecular weight is 7698 g/mol.

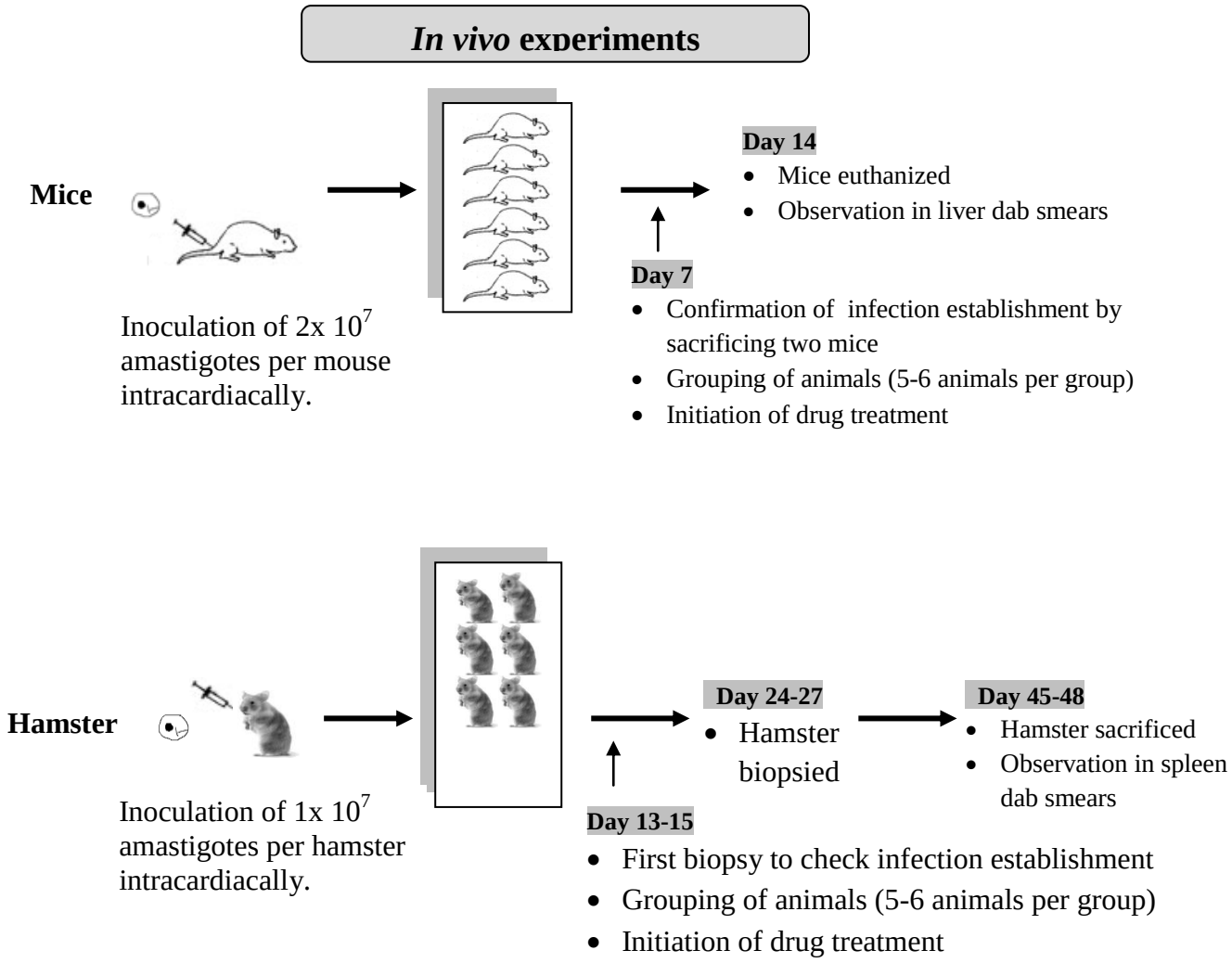
1.4. Miltefosine

Miltefosine was purchased from Synpha*B*ase AG (Switzerland). For the *in vivo* part of the study, miltefosine was dissolved in deionized water.

1.5. Liposome preparation

Liposomes containing CpG ODN were prepared by the dehydration-rehydration vesicle (DRV) method (Jaafari *et al.*,2007; Kirby and Gregoriadis,1984).The lipid phase consists of 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (6 µM), and cholesterol (2 µM) dissolved in chloroform: methanol (2:1, v/v) in a flat bottom tube. The solvent was removed by slow evaporation under nitrogen in deposition of a thin film of lipid on the tube surface. The tube was dried to remove any trace of solvent. The lipid film was then hydrated and dispersed in a solution of appropriate quantity of CpG ODN in distilled water and processed to get the desired preparation of liposomal CpG ODN. The percent incorporation of CpG into the liposomes was determined using UV absorption at 260 nm and the concentration of CpG ODN in the liposomes was adjusted to the desired concentration prior to administration.

1.6. Experimental Plan



1.7. Infection of mice and antileishmanial efficacy evaluation

BALB/c mice were infected intracardiacally with 2x10^7*L. donovani* amastigotes and randomly sorted into groups of five or six animals each. The same number of mice was kept as untreated control group. Mice were dosed by ip (intra peritoneal) and po (*per os*) routes at 7^{th} day post infection for 5 consecutive days. Animals were sacrificed on day 3 post treatment (day 14 post infection). Impression smears of livers were prepared, fixed with 100% methanol, and stained with 20% Giemsa stain for 45 min. The number of amastigotes per 500 liver cell nuclei was determined. The percent inhibition (PI) was calculated for all drug-treated groups in relation to untreated group.

1.8. Infection of hamster and antileishmanial efficacy evaluation

The evaluation in hamsters was carried out in accordance with the method described by Bhatnagar *et al.* (1989). Five or six animals were used for each agent and the same numbers were kept as untreated controls. The drug treatment was given by ip and po routes. To assess the effect of drugs, spleen biopsy was performed on each animal on 7^{th} day post treatment and amastigote counts were assessed by Giemsa staining (Gupta *et al.*,2005).The PI was calculated for all drug-treated groups in relation to untreated group.

1.9. Optimization of CpG ODN and miltefosine dose regimens against *L.donovani*/mouse and hamster model

Each group consisting of five or six infected animals in two replicates with each species were used. The CpG ODN was administered in doses viz. 0.5, 1, 2, 5 nM prepared in deionized water by ip route for dose optimization. A 1nM dose of CpG ODN was found to be most appropriate for combination studies in both the species. Miltefosine was given at doses ranging from 20mg/kg to 1.25 mg/kg in mice and 40 mg/kg to 2.5 mg/kg in hamsters for 5 days by po route to select the sub curative dose. The sub-curative dose selected was 2.5 mg/kg and 5 mg/kg in mice and hamster, respectively.

1.10. Evaluation of free CpG ODN and liposomal CpG ODN alone and in combination with miltefosine in *L.donovani*/mouse and hamster model

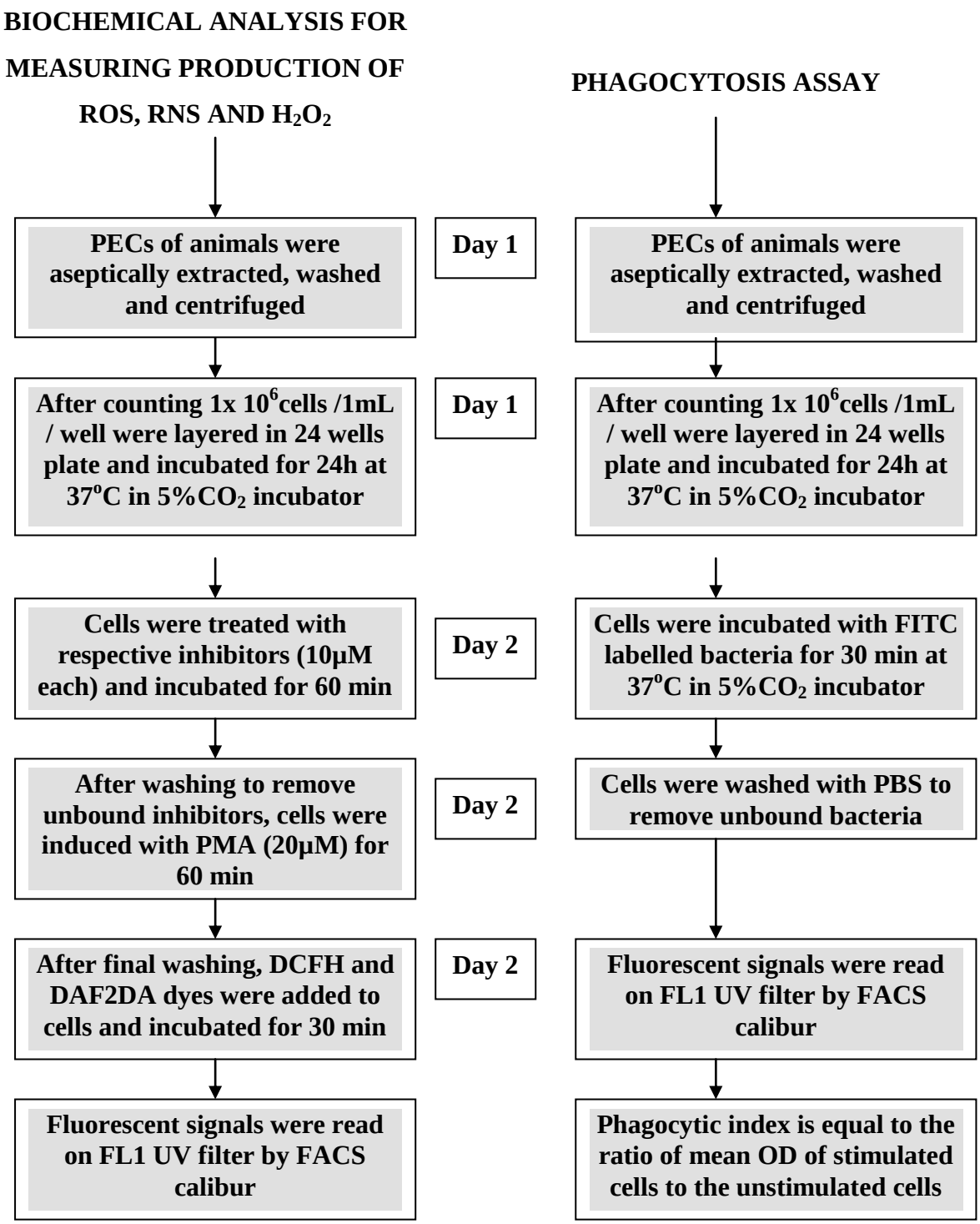
Seven groups of mice each consisting of five or six animals in two replicates were used for these experiments. Mice of Group I received free CpG ODN (1 nM) by ip route, Group II received free CpG ODN (1 nM) + miltefosine (2.5mg/kg x 5d), Group III received liposomal CpG ODN (1 nM), Group IV received liposomal CpG ODN (1 nM) + miltefosine(2.5mg/kg x 5d), Group V and VI received sub-curative (2.5 mg/kg) and effective miltefosine (10 mg/kg) po for 5 days respectively, Group VII receiving PBS served as a control. The same protocol was adopted for hamsters, where miltefosine was given at 5mg/kg x 5 (sub curative)

and 20 mg/kg x 5 (effective) doses. The PI was calculated for all drug-treated groups in relation to a non treated group.

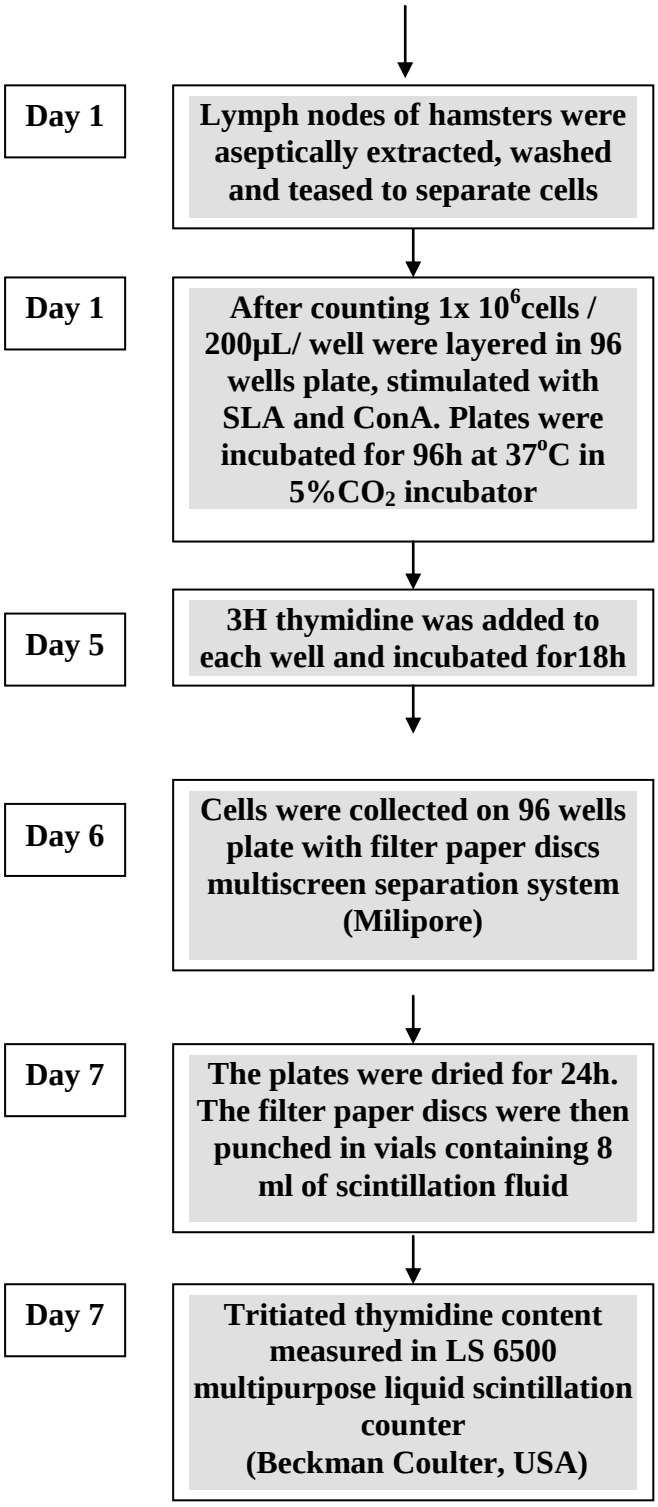
1.11. Immunological and Biochemical Analysis

- Reactive oxygen species production assay using inhibitor pentoxifylline (PTx)
- Hydrogen peroxide production assay using inhibitor sodium azide (NaN_3)
- Reactive Nitrogen species production assay using inhibitor N-nitro-L-arginine methyl ester (L-NAME)
- Phagocytosisassay
- Lymphocyte proliferation assay

Immunological & Biochemical Analysis in *L.donovani*/mouse and hamster model



LYMPHOCYTE PROLIFERATION ASSAY
(*L.donovani*/hamster model)



1.12. Evaluation of Th1/Th2 cytokines in *L.donovani*/mouse model

Serum samples from animals of treated and untreated control groups were analysed for various Th1 [Tumor necrosis factor (TNF), IFN-γ, IL-2] and Th2 (IL-4, IL-5) cytokines by CBA mouse Th1/Th2 cytokine assay kit (BD Biosciences) in accordance with the manufacturer's instructions. Kit is provided with five bead populations with distinct fluorescence intensities which have been coated with capture antibodies specific for IL-4, IL-5, IFN-γ, TNF and IL-2 proteins. The recombinant standards and samples mixed with the cytokine capture beads and incubated at room temperature for 2h (protected from light) with PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine. Samples were read on FACS Calibur having BD FACS Comp software. Data were evaluated in five parameters viz. FSC, SSC, FL1, FL2, FL3 and presented as mean fluorescent signals.

1.13. Statistical Analysis

Results are presented as mean ± S.D. of two experiments and analysis of data is carried out by Bonferroni's multiple comparison tests and Dunnett's multiple comparison tests. Differences with $P< 0.05$ were considered significant. Sub-curative dose was determined by probit analysis (Finney, 1971).

2. RESULTS

A. CpG ODN 1826

2.1. Dose optimization study for CpG ODN in *L.donovani*/mouse and hamstermodel

Results of CpG dose optimization have been displayed in Fig.1.

(i) In mouse model:

Various doses of CpG ODN was explored and best antileishmanial efficacy was witnessed at adose of 1 nM / single shot, ip (41.1% inhibition in parasite multiplication) followed by gradually decreasing efficacy with 2 and 5 nM doses namely 22% and 18% inhibition in parasite multiplication, respectively. 0.5nM dose was also tested but it showed no efficacy (11.2%). Based on the results, 1 nM / single shot, ip was selected for combination trial.

(ii) In hamster model:

Best antileishmanial efficacy was observed at a dose of 1 nM / single shot / ip, i.e. 55.7% inhibition in parasite multiplication followed by gradually decreasing efficacy at 0.5, 2 and 5 nM doses, namely 22%, 38.1% and 33.3 % inhibition in parasite multiplication, respectively.

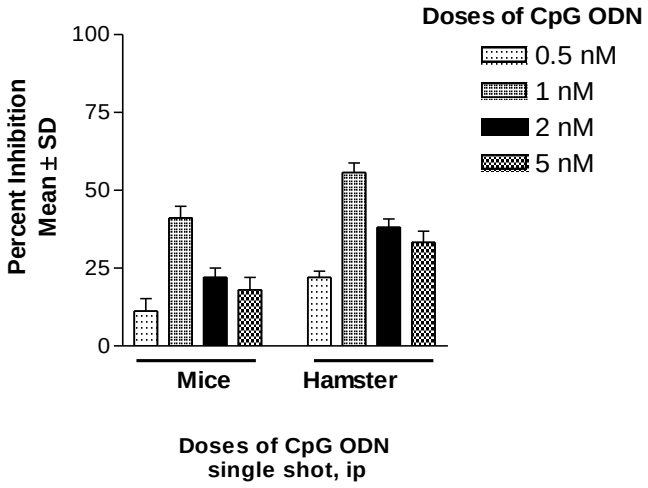


Fig.1 Dose optimization of CpG ODN

Legend.1*Leishmania donovani* infection was given to BALB/c mice ($2x10^7$amastigotes/animal)and hamsters($1x10^7$amastigotes/animal) intracardically. Mice and hamsters were dosed at 7 and 15 days post infection respectively with single shot of various dosages of CpG ODN by intraperitoneal route. Mice were

sacrificed 3 days after the completion of treatment where as hamsters were sacrificed 7 days after the completion of treatment. Mean P.I. ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.2 Effect of liposome preparation on antileishmanial efficacy of CpG ODN

Results have been presented in Fig.2. A dose of 1nM of CpG ODN was administered in mice and hamsters in free and liposomal form. Additionally same number of animals was also treated with empty vehicle to analyse the effect of liposome preparation. It was observed that liposomal encapsulation significantly enhanced the antileishmanial efficacy of CpG ODN from 42% to 55% and 48% to 60% in mice and hamster respectively. However empty vehicle failed to exhibit any effect on parasitic inhibition.

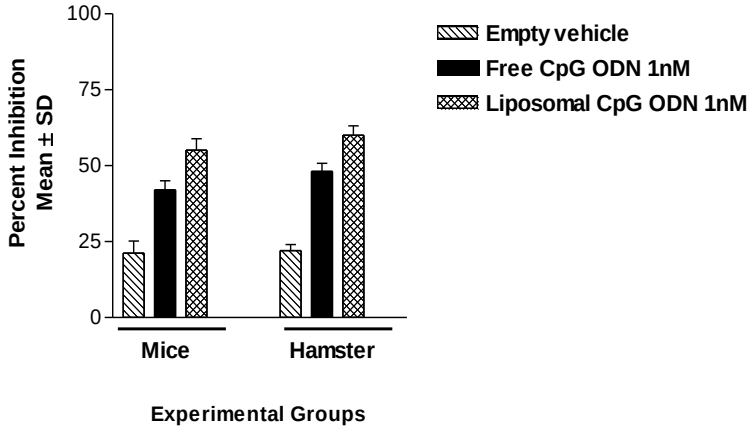


Fig.2 Effect of liposome preparation

Legend.2*Leishmania donovani* infection was given to BALB/c mice ($2x10^7$amastigotes/animal)and hamsters($1x10^7$amastigotes/animal) intracardically. Mice and hamsters were dosed at 7 and 15 days post infection respectively with single shot of 1nM of free and liposomal CpG ODN by intraperitoneal route. Mice were sacrificed 3 days after the completion of treatment where as hamsters were sacrificed 7 days after the completion of treatment. Mean P.I. ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.3. Dose optimization of miltefosine in *L.donovani*/mouse and hamster model

Results of miltefosine dose optimization in mouse and hamster have been presented in Fig.3.

(i) In mouse model:

Miltefosine was evaluated at various doses ranging from 20mg/kg to 1.25mg/kg for 5 days by po route in order to select the sub curative dose. Parasite inhibition observed at 20mg/kg was 98% followed by 90%, 70.5%, 48.8% and 34.5% at 10, 5, 2.5, 1.25 mg/kg doses respectively. Best dose for combination trial was selected as 2.5mg/kg.

(ii) In hamster model:

Miltefosine was tested at various doses ranging from 40mg/kg to 2.5mg/kg for five days by po route. Parasite inhibition observed at 40mg/kg was 99% followed by 92%, 80.3%, 55.5% and 35.8% at 20, 10, 5, 2.5 mg/kg doses respectively. Best dose for combination trial was selected as 5mg/kg as it was sub curative and non-toxic.

Fig.3 Dose optimization of miltefosine

Legend.3 *Leishmania donovani* infection was given to BALB/c mice ($2x10^7$amastigotes/animal)and hamsters($1x10^7$amastigotes/animal) intracardically. Mice and hamsters were dosed at 7 and 15 days post infection respectively with various dosages of miltefosine by oral route for five days. Mice were sacrificed 3 days after the completion of treatment where as hamsters were sacrificed 7 days after the completion of treatment. Mean P.I. ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.4. Combination therapy (CpG ODN+ Miltefosine) in *L.donovani*/mouse and hamster model

Fig.4 displayed the results of combination therapy of free and liposomal CpG ODN with miltefosine.

(i) In mouse model:

Free CpG ODN has shown an efficacy of 42% which was moderately enhanced to 55.2% by liposomal encapsulation ($P<0.01$). Miltefosine alone exhibited parasite inhibition of 46%. However, when free CpG ODN given with sub-curative dose of miltefosine, parasite inhibition increased from 46% to 65% ($P<0.01$) and liposomal CpG ODN further enhanced the efficacy up to 85% ($P<0.001$). Interestingly, the effectiveness of this combination was very close to the effective dose (10 mg/kg for 5days) of the miltefosine (90.2%).

(ii)In hamster model:

Free CpG ODN showed an efficacy of 32.3% which was moderately enhanced to 50.9% by liposomal encapsulation ($P<0.01$). Miltefosine alone exhibited parasite inhibition (PI) of 50%. However, when free CpG ODN given with suboptimal dose of miltefosine, parasite inhibition increased from 50% to 63.1% ($P<0.001$) and liposomal CpG ODN further enhanced the efficacy up to 81.7% ($P<0.001$). The efficacy of this combination was close to the efficacy of effective dose (20 mg/kg for 5days) of the miltefosine (91.8%).

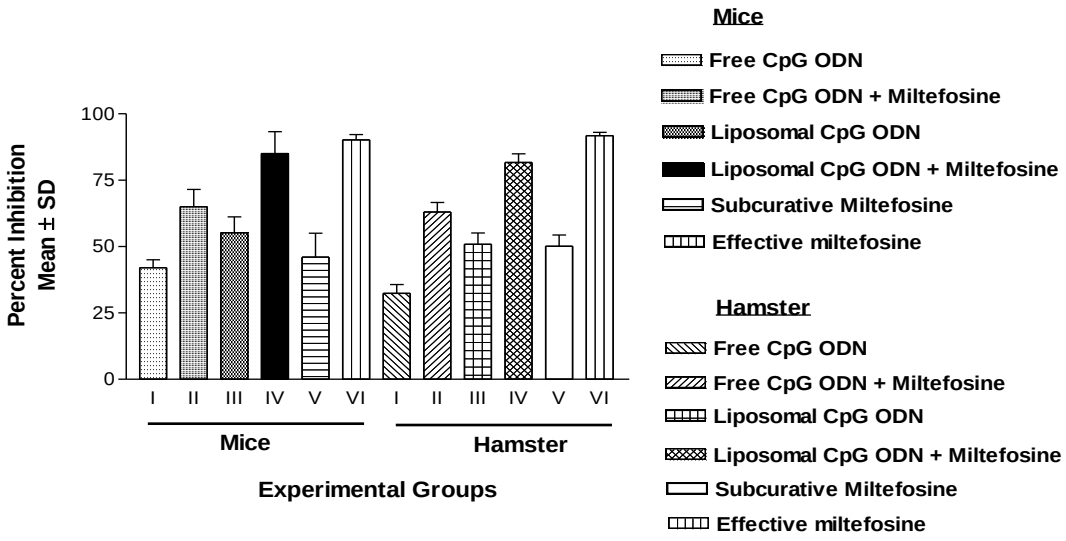
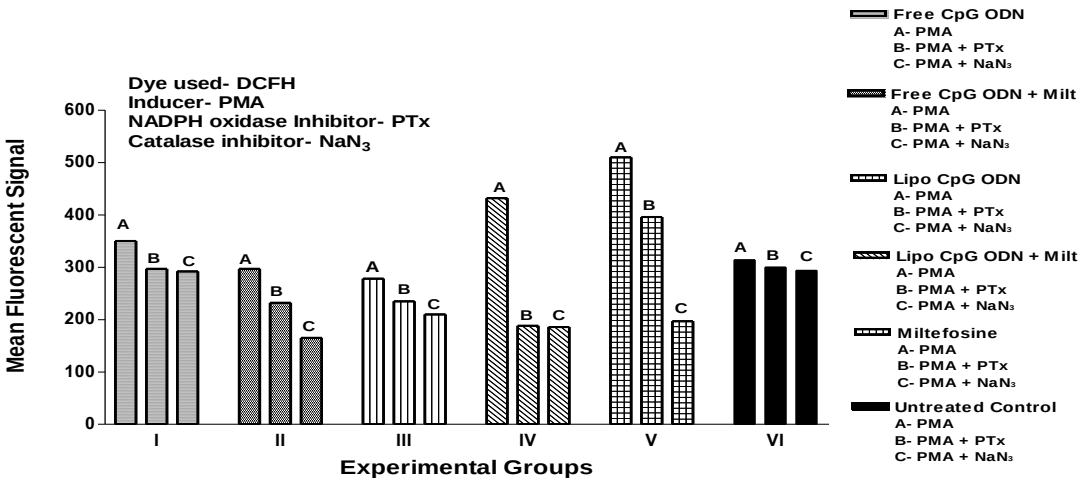


Fig.4 Combination therapy of free and liposomal CpG ODN 1826 with miltefosine

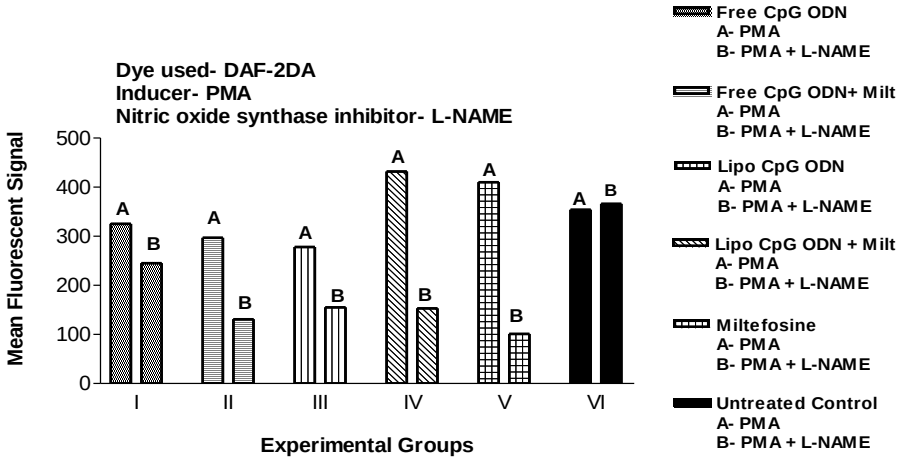
Legend.4 Mean PI ± S.D. was calculated by comparing parasitic burden of treated groups to control animals. Significance among different groups was calculated by Bonferroni's multiple comparison tests. Significance in case of mice: (I vs II – P<0.001; I vs III- P< 0.01; II vs IV- P< 0.001); (V vs II – P< 0.01; V vs IV- P< 0.001). Significance in case of hamsters: (I vs II – P<0.001; I vs III- P<0.01; II vs IV- P< 0.001); (V vs II – P < 0.01; V vs IV- P< 0.001).

2.5. Biochemical assays in *L.donovani*/mouse model

The results have been displayed in Fig.5 (a, b). Mice administered with free CpG ODN showed a moderate NO, ROS and H_2O_2 production (P<0.05). Free CpG ODN when co-administered with miltefosine, enhanced the production of NO and H_2O_2 (P<0.01) and ROS (P<0.05). Liposomal CpG ODN showed significant NO production (P<0.01) and moderate ROS and H_2O_2 production (P<0.05). However, when it was combined with miltefosine, remarkable production of NO, ROS and H_2O_2 (P<0.001) was observed. Miltefosine showed significant NO, H_2O_2 production (P<0.001) and a moderate ROS production (P<0.01).



(a) Biochemical assay for production of ROS and H_2O_2 in mouse



(b) Biochemical assay for production of RNS in mouse

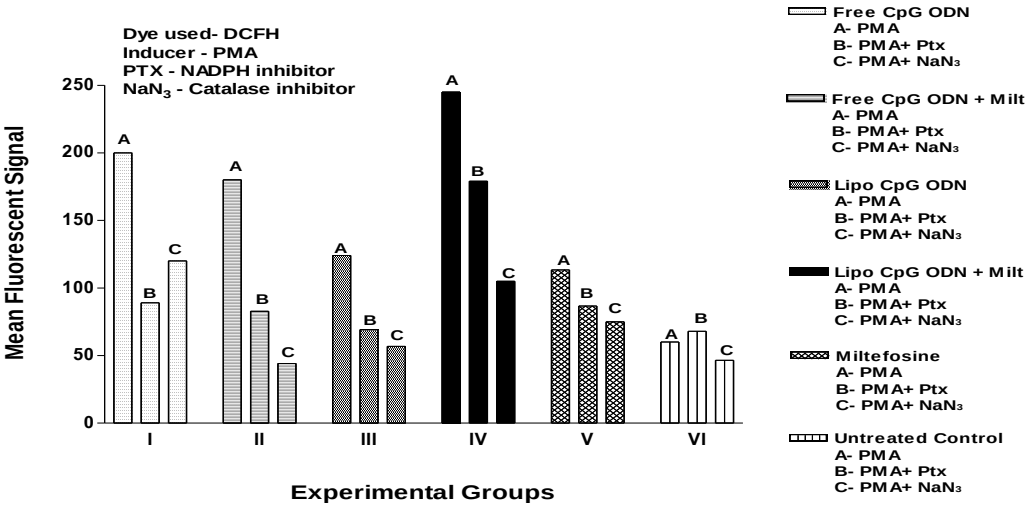
Fig.5 Biochemical assay for production of ROS, RNS and H_2O_2

Legend.5 Significance of inhibition of different treated groups was assessed against untreated (control) animals by Dunnett's multiple comparison tests. For ROS: (VI vs I – P<0.05; VI vs II – P<0.05; VI vs III– P<0.05; VI vs IV– P<0.001 ; VI vs V – P<0.01).For H_2O_2: (VI vs I – P<0.05; VI vs II – P<0.01; VI vs III – P<0.05; VI vs IV– P<0.001 ; VI vs V – P<0.001). For RNS: (VI vs I – P<0.05; VI vs II – P<0.01; VI vs III – P<0.01; VI vs IV– P<0.001 ; VI vs V – P<0.001).

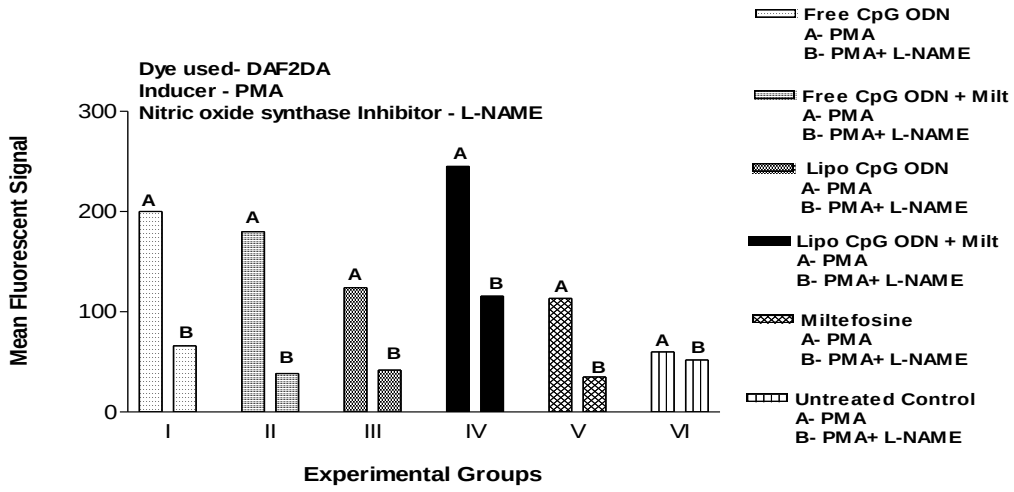
2.6. Biochemical assays in *L.donovani*/hamster model

The results have been displayed in Fig.6 (a, b). Animals administered with free CpG ODN showed a moderate ROS, H_2O_2 and NO production (P<0.01). Free CpG ODN, when co-administered with miltefosine, it significantly enhanced production of H_2O_2 and NO

(P<0.001). Liposomal CpG ODN alone and in combination with miltefosine also showed remarkable production of ROS, H_2O_2 and NO (P<0.001). Miltefosine alone resulted in significant NO production (P<0.001).



(a) Biochemical assay for production of ROS and H_2O_2 in hamsters.



(b) Biochemical assay for production of RNS in hamsters.

Fig.6 Biochemical assay for production of ROS, RNS and H_2O_2

Legend.6 Significance of inhibition of different treated groups was assessed against untreated (control) animals by Dunnett's multiple comparison tests For ROS: (VI vs I – P<0.01; VI vs II – P<0.001; VI vs III– P<0.001; VI vs IV– P<0.001 ; VI vs V – P<0.01).For H_2O_2: (VI vs I – P<0.01; VI vs II – P<0.001; VI vs III – P<0.01; VI vs

IV– $P<0.001$; VI vs V – $P<0.01$). For RNS: (VI vs I – $P<0.01$; VI vs II – $P<0.001$; VI vs III – $P<0.01$; VI vs IV– $P<0.001$; VI vs V – $P<0.001$).

2.7. Phagocytosis in *L.donovani*/mouse and hamster model

Results of phagocytosis have been presented in Fig.7.

(i) In mouse model:

Cells of untreated control mice showed lowest phagocytic index (12.5 ± 9.0). Treatment with miltefosine moderately increased phagocytic index to 29.4 ($P<0.01$). Mice treated with free CpG ODN also showed similar index 30.0 ± 5.0 ($P<0.01$). Free CpG ODN when co-administered with miltefosine, enhanced the index to 45.5 ± 5.0 ($P<0.001$). Liposomal CpG ODN gave phagocytic index of 37.5 ± 4.0 ($P<0.01$) and when given with miltefosine there was a remarkable increase in index up to 55.1 ± 3.0 ($P<0.001$), which was almost equal to phagocytic index of normal uninfected animals (60 ± 3.0).

(ii) In hamster model:

Cells of untreated control hamster showed lowest phagocytic index (17 ± 5.0). Miltefosine treated animals exhibited moderately increased index of 36.5 ± 13.2 ($P<0.01$). Hamster treated with free CpG ODN showed phagocytic index of 40.2 ± 7.9 ($P<0.01$) and when co-administered with miltefosine, the index was enhanced to 46.5 ± 9.9 ($P<0.01$). Liposomal CpG ODN showed phagocytic index of 65.2 ± 5.5 ($P<0.01$) and, when given with miltefosine, remarkable increase in index up to 78.3 ± 5.2 ($P<0.001$) was observed, which was almost equal to phagocytic index of normal uninfected animals (88 ± 5.0).

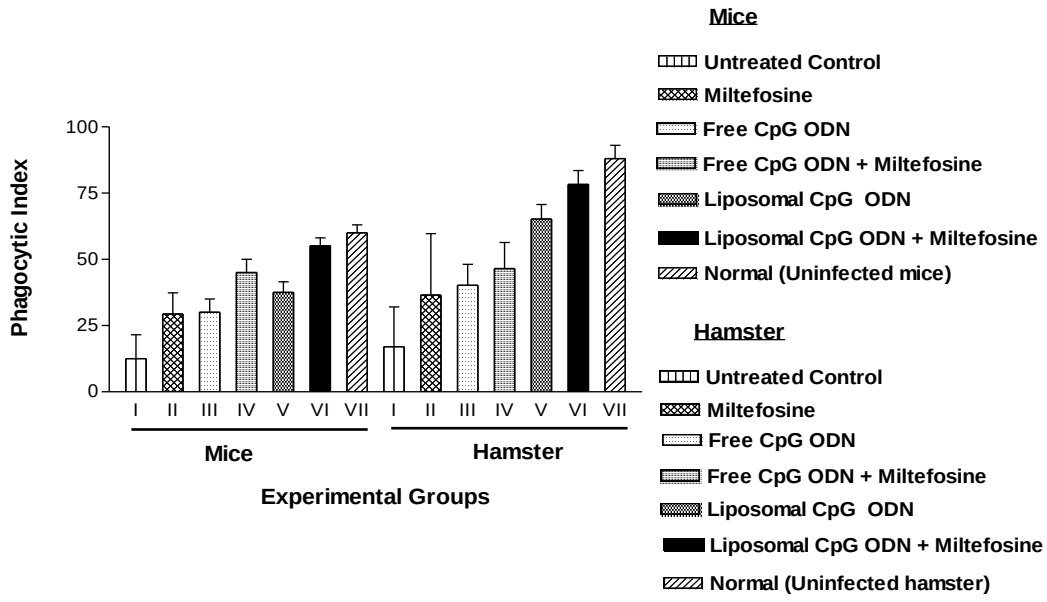


Fig.7 Phagocytosis assay

Legend.7. Fluorescence of stimulated and unstimulated cells of each group compared and significance of activity of different treated groups was assessed against untreated (control) animals by Dunnett's multiple comparison tests. Significance in case of mice: (I vs II – $P<0.01$; I vs III – $P<0.01$; I vs IV – $P<0.001$; I vs V– $P<0.01$; I vs VI – $P<0.001$; I vs VII – $P<0.001$). Significance in case of hamster: (I vs II – $P<0.01$; I vs III – $P<0.05$; I vs IV – $P<0.01$; I vs V– $P<0.01$; I vs VI – $P<0.001$; I vs VII – $P<0.001$).

2.8. CMI response in *L.donovani*/mouse model

From the results displayed in Fig.8, more than a 3-fold rise in TNF (tumour necrosis factor) & IL2 production and a moderate rise in IFNγ production were observed after liposomal encapsulation of CpG ODN. Free CpG ODN when combined with miltefosine, gave a 2-fold rise in both TNF and IFNγ. Significant enhancement in IFNγ production was witnessed in animals co-administered with liposomal CpG ODN and miltefosine. Briefly, we can say that the combination therapy involving free or liposomal CpG ODN with miltefosine increased Th1 (TNF, IFNγ, IL2) cytokine levels and down regulated Th2 (IL4, IL5) cytokines. Animals treated with miltefosine alone showed moderately enhanced Th1 response. Untreated control group, however, did not show any increase in Th1 response.

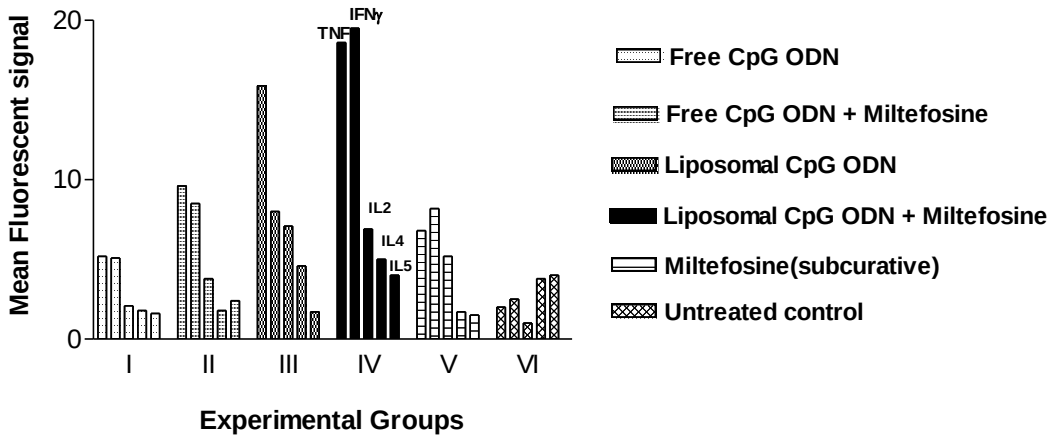


Fig.8 Th1/ Th2 cytokine assay

Legend.8 Serum samples from animals of treated and untreated control groups were analysed for various Th1 (TNF, IFNγ, IL2) and Th2 (IL4 and IL5) cytokines by FACS calibur having BD FACS Comp software. Data were evaluated in five parameters viz. FSC, SSC, FL1, FL2 and FL3 and presented as mean fluorescent signals.

2.9. Lymphocyte proliferation test in *L.donovani*/hamster model

Detailed results have been depicted in Fig.9. As compared to untreated control (SI: 52.5 ± 23.2), animals treated with combination of liposomal CpG ODN and miltefosine showed remarkable cell mediated immune response (SI: 158.3 ± 5.2) (P<0.001). Moderately significant CMI responses was recorded in animals treated with free CpG ODN (SI: 80.2 ± 7.9), free CpG ODN + Miltefosine (111.0 ± 7.8), liposomal CpG ODN (SI: 98.5 ± 9.9) and miltefosine (SI: 105.2 ± 5.5).

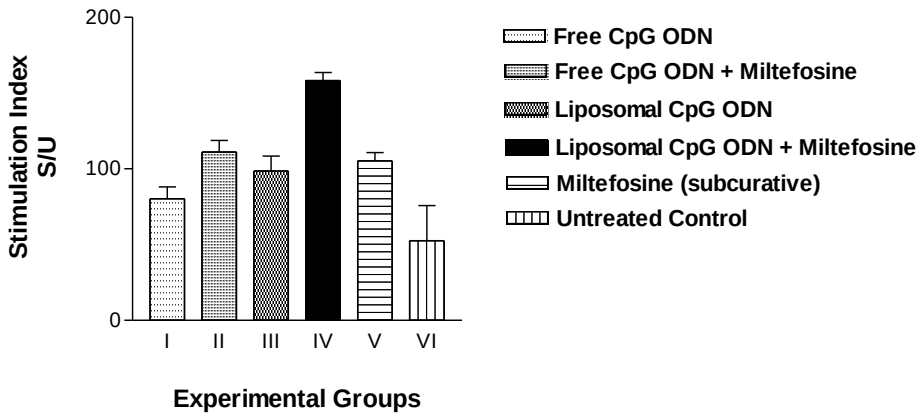


Fig.9 Lymphocyte proliferation assay

Legend.9 Proliferation of cells was read on PRIAS CLD / 400 liquid Scintillation Counter and Stimulation Index were calculated. Significance of activity of different treated groups was assessed against untreated (control) animals by Dunnett's multiple comparison tests. (I vs II – $P<0.05$; I vs III – $P<0.01$; I vs IV – $P<0.01$; I vs V– $P<0.001$; I vs VI – $P<0.01$).

B. CpG ODN 2006

2.10 Dose optimization of CpG ODN 2006 in *L.donovani*/mouse and hamster model

Results of CpG dose optimization in mouse and hamster have been displayed in Fig.10.

(i) In mouse model:

Of the various doses of CpG ODN tried, the best antileishmanial efficacy was witnessed at a dose of 1 nM / single shot, ip (45.1% inhibition in parasite multiplication). This was followed by gradually decreasing efficacy with 2 and 5 nM doses namely 27% and 16% inhibition in parasite multiplication, respectively. 0.5nM dose was also tested but it showed no efficacy (21.2%). Based on the results, 1 nM / single shot, ip was selected for combination trial.

(ii) In hamster model:

Best antileishmanial efficacy was observed at a dose of 1 nM / single shot / ip, i.e. 52% inhibition in parasite multiplication followed by gradually decreasing efficacy at 0.5, 2 and 5 nM doses, namely 25%, 32% and 28 % inhibition in parasite multiplication, respectively.

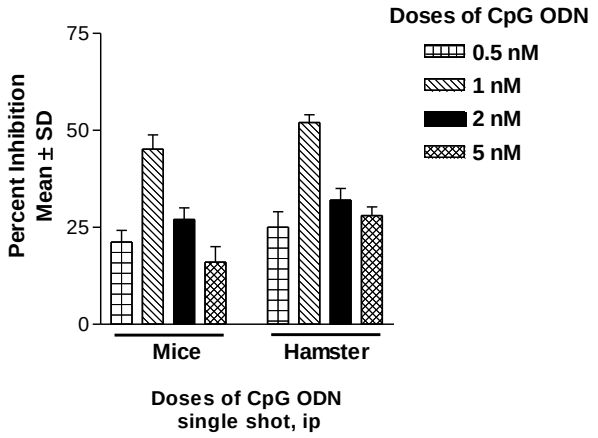


Fig.10 Dose optimization of CpG ODN 2006

Legend.10*Leishmania donovani* infection was given to BALB/c mice ($2x10^7$amastigotes/animal) and hamsters ($1x10^7$amastigotes/animal) intracardically. Mice and hamsters were dosed at 7 and 15 days post infection respectively with single shot of various dosages of CpG ODN by intraperitoneal route. Mice were sacrificed 3 days after the completion of treatment where as hamsters were sacrificed 7 days after the completion of treatment. Mean P.I. ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.11. Combination therapy (CpG ODN+ Miltefosine) in *L.donovani*/mouse and hamster model

The results of combination therapy of free and liposomal CpG ODN with miltefosine have been displayed in Fig.11.

(i) In mouse model:

Free CpG ODN showed an efficacy of 40% which was moderately enhanced to 51% by liposomal encapsulation ($P<0.01$). Parasite inhibition (PI) of miltefosine at 2.5 mg/kg and 5 mg/kg were 44% and 68.3% respectively. However, when free CpG ODN given with suboptimal dose (2.5 mg/kg) of miltefosine, parasite inhibition increased from 44% to 72.4% ($P<0.001$) and liposomal CpG ODN further enhanced this efficacy up to 90.6% ($P<0.001$). Parasitic inhibition was markedly increased to 97.1% when liposomal CpG ODN was administered with 5 mg/kg of miltefosine. The efficacy of this combination was comparable with the efficacy of curative dose (20 mg/kg for 5days) of the miltefosine (98%).

(ii) In hamster model:

Free CpG ODN has shown an efficacy of 46.8% which was moderately enhanced to 55.1% by liposomal encapsulation (P<0.01). Parasite inhibition (PI) of miltefosine at 5 mg/kg and 10 mg/kg were 50% and 70.1% respectively. However, when free CpG ODN given with sub-curative dose (5 mg/kg) of miltefosine, parasite inhibition increased from 50% to 68.5% (P<0.01) and liposomal CpG ODN further enhanced the efficacy up to 88.5% (P<0.001). Interestingly, the effectiveness of this combination was further enhanced to 96% when liposomal CpG ODN was co-administered with 10 mg/kg of miltefosine. This efficacy was close to the curative dose (40 mg/kg for 5days) of the miltefosine (99%).

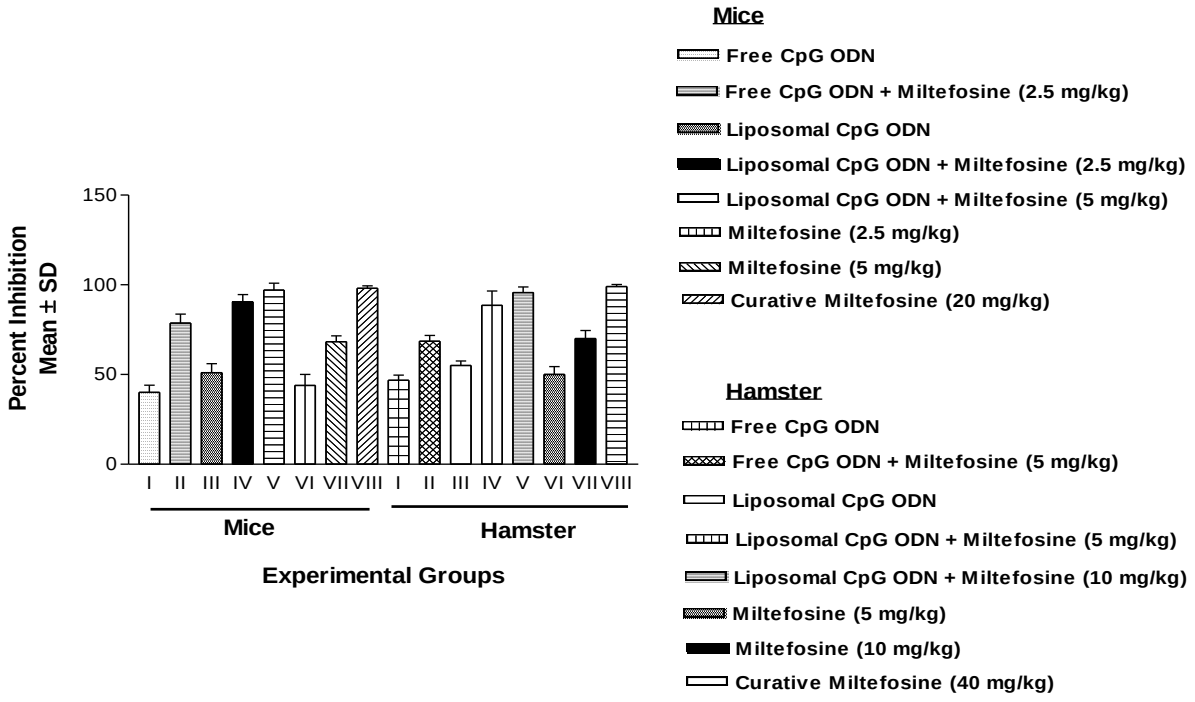


Fig.11 Combination therapy of free and liposomal CpG ODN 2006 with miltefosine

Legend.11 Mean PI ± S.D. was calculated by comparing parasitic burden of treated groups to control animals. Significance among different groups was calculated by Bonferroni's multiple comparison tests. Significance in case of mice: (I vs II – P< 0.001; I vs III- P< 0.01; II vs IV- P< 0.001;II vsVI- P< 0.001;III vs IV-P< 0.001;III vs V-P< 0.001;IV vs VI – P< 0.001; V vsVII- P< 0.001). Significance in case of hamsters: : (I vs II – P< 0.001; I vs III- P< 0.01; II vs IV- P< 0.001;II vsVI- P< 0.01;III vs IV-P< 0.001;III vs V-P< 0.001;IV vs VI – P< 0.001; V vsVII- P< 0.001).

3. DISCUSSION

In view of the severe immunosuppression in VL, a rational approach to effectively combat the parasitic scourge would be to enhance the immune status of the host (Murray, 1988; Badaro *et al.,*1994).Use of CpG ODN has been reported in field of leishmaniasis especially as immunomodulators and adjuvant with various immunogens (Verthelyi and Klinman,2003; Datta *et al.,*2003; Badiee *et al.,*2008; Iborra *et al.,* 2008).Keeping this in mind, we have explored the adjunct effect of fully thioated CpG ODNs (free and liposomal) on the efficacy of miltefosine using *L.donovani*/ BALB/c mouse and hamster model. It was observed that the efficacy profile of CpG ODN 1826 was in corroboration with efficacy of CpG ODN 2006. Detailed immunological and biochemical alterations were studied with CpG ODN 1826 only. Results clearly show that liposomal encapsulation enhanced antileishmanial efficacy of free CpG ODN. Co-administered liposomal CpG ODN and miltefosine showed a better inhibitory effect than free CpG ODN+ miltefosine, free CpG ODN alone or miltefosine alone. In order to explore the effect of liposomal lipids on parasitic inhibition, an empty liposome preparation was also administered along with free and liposomal CpG ODN (1nM) in a separate experiment. We have not observed any improvement over the untreated infected control groups. This was in agreement with the previous report of Badiee *et al.* (2008).

Results of immunological assays in mice suggested that CpG ODN 1826 potentiated the cell mediated immunity, evident from significant rise in Th1 cytokines and down regulation of Th2 cytokines. Liposomal encapsulation of CpG ODN enhanced IL-2, TNF and IFNγ levels. In biochemical assay, liposomal CpG ODN combined with miltefosine resulted in remarkable production of NO, ROS and H_2O_2. Thus, the promising antileishmanial efficacy of liposomal CpG ODN + miltefosine is strongly supported by enhancement of Th1 cytokines as well as NO, ROS and H_2O_2 levels. In order to strengthen the study, CpG ODN 2006 was also evaluated with slightly higher sub-curative doses of miltefosine namely at 5 mg/kg and

10mg/kg for five days in mice and hamsters respectively. This combination significantly enhanced the efficacy of miltefosine in both the rodent models (in case of mice 90.6% to 97% and in case of hamsters 88.5% to 96%) compared to the combination using sub-curative dose of miltefosine. The efficacy of this combination was comparable with the efficacy of the curative dose (98% at 20 mg/kg in case of mice and 99% at 40 mg/kg in case of hamsters) of the miltefosine.Thus this study clearly offers the scope for use of CpG ODN in combination with miltefosine for the treatment of human VL.

CHAPTER-6

IMMUNOMODULATORY EFFECT OF PICROLIV ON THE EFFICACY OF PAROMOMYCIN AND MILTEFOSINE IN COMBINATION

In the present work, a rational approach which can modulate the immune response to overcome the negative control systems and to boost the positive killing responses was adopted. This study was designed to investigate the immunomodulatory effect of picroliv (standardized fraction from the alcoholic extract of root and rhizome of *Picrorhiza kurroa)* on a combination of paromomycin and miltefosine using *L.donovani*/hamster model. The hamster model is known to closely mimic the condition of human VL better than any other animal model (Melby *et al.,* 2001). Along with this, *in vitro* assays like measurement of mitochondrial potential drop and apoptotic profiling was performed in drug treated promastigotes. To further strengthen the study, *in vivo* assays like measurement of production of toxic oxygen and nitrogen metabolites (ROS, RNS, H_2O_2), lymphocyte proliferation test, and phagocytosis were also studied in treated and untreated hamsters.

1. MATERIALS AND METHODS

1.1. Parasite

L. donovani (MHOM/IN/80/Dd8)

1.2. Compound

Paromomycin sulphate was received from Sigma- Aldrich, India, miltefosine was purchased from Synpha*B*ase AG, Geneva, Switzerland and picroliv (powder) was obtained from Medicinal Chemistry and Process division of CDRI, Lucknow, India. Picroliv, paromomycin sulphate and miltefosine were dissolved in deionized water.

1.3. Animals

Inbred hamsters (40-45 g) of both sexes were used for the study.

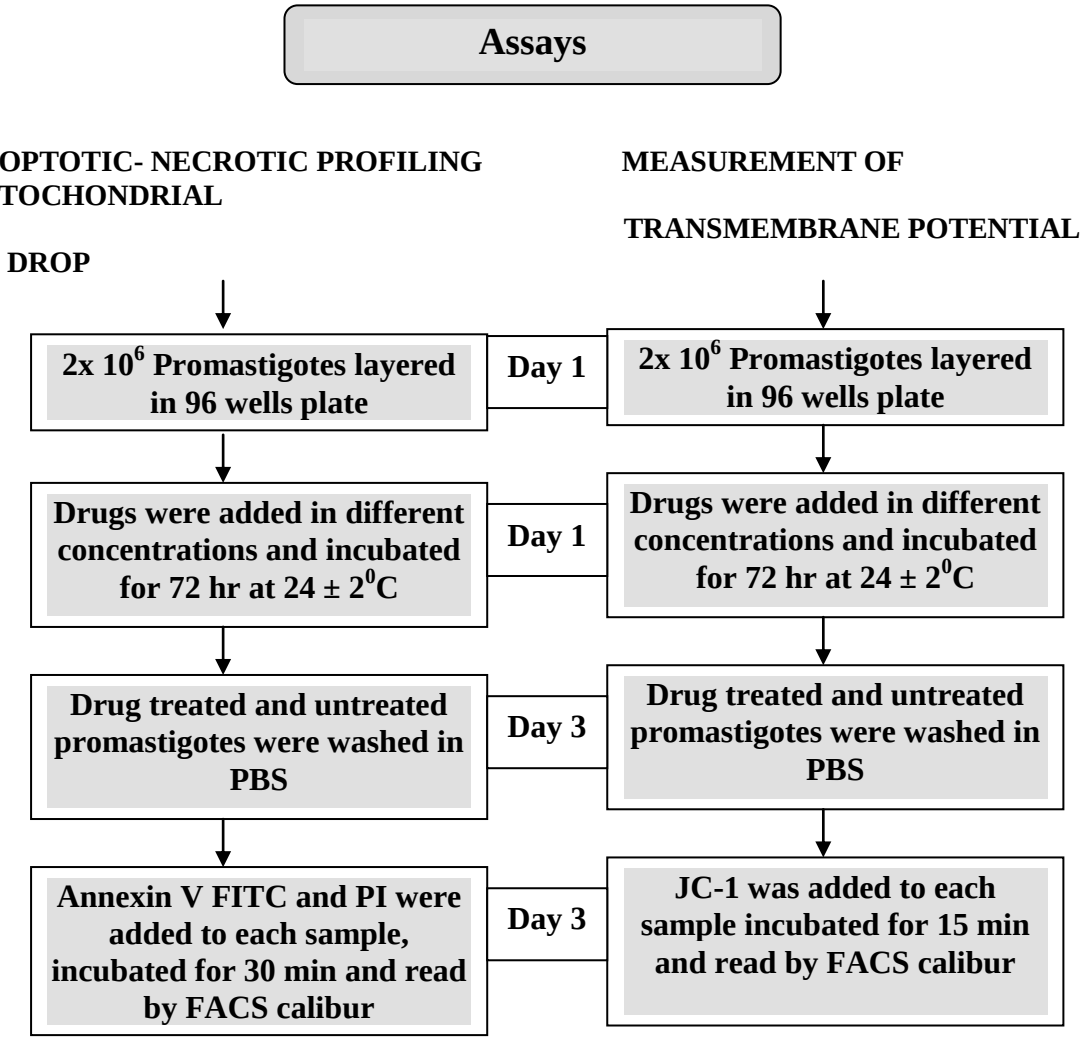
1.4. Experimental plan

1.4.1. *In vitro* evaluation of drug responses using promastigote and amastigote assay

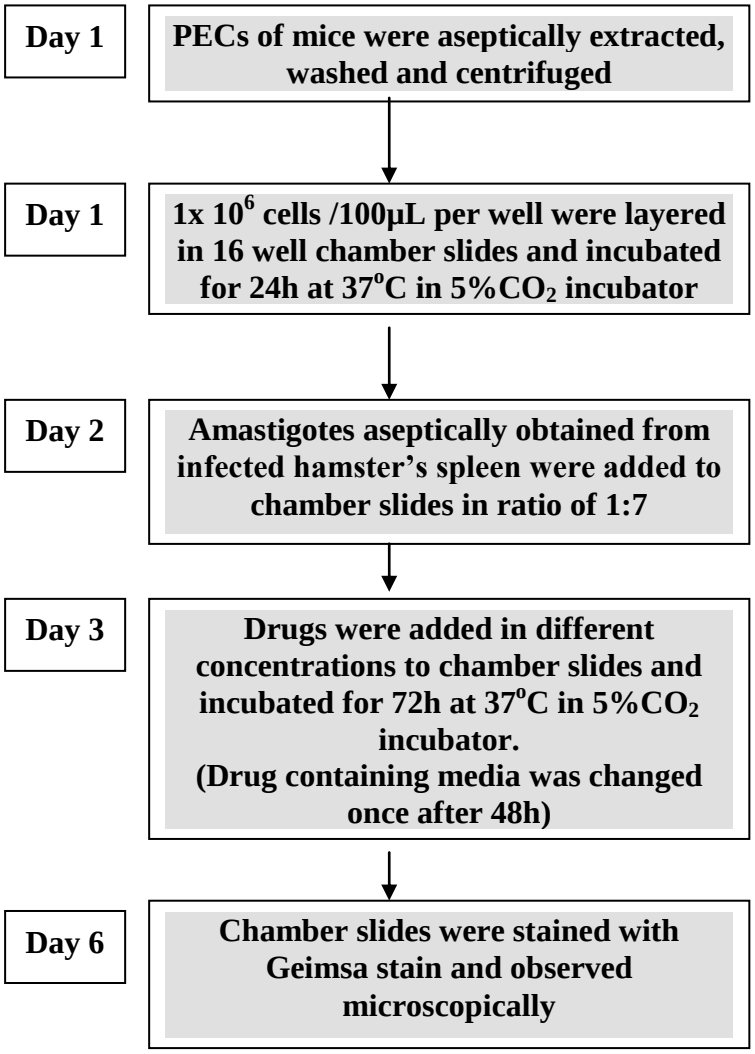
In order to assess antileishmanial efficacy of miltefosine, paromomycin and picroliv they were tested against promastigotes and intracellular amastigotes at various concentrations.

Promastigotes were used to study apoptosis and drop in mitochondrial membrane potential whereas intra-macrophagic amastigotes were used to evaluate drug interaction at sub-curative doses. Drug concentrations of miltefosine, paromomycin and picroliv used for these assays were 6.25 µM, 50 µM and 250 µM respectively.

- ***In vitro* assays using promastigotes**



- ***In vitro* assay using intra-macrophagic amastigotes**



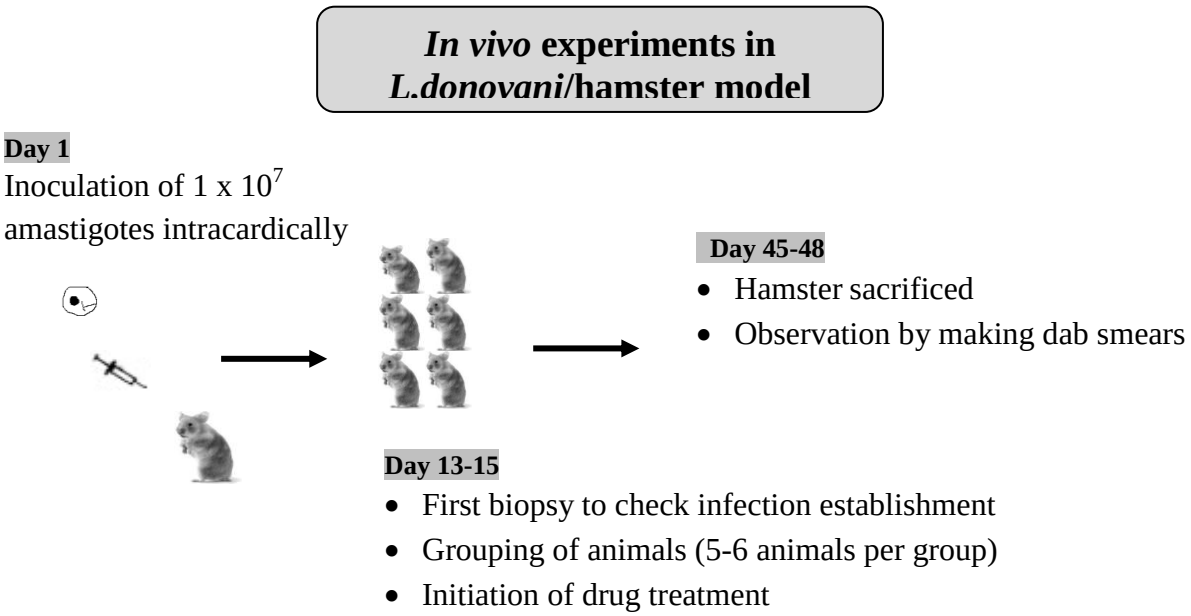
1.4.2. *In vivo* efficacy evaluation using *L.donovani*/hamster model

The evaluation in hamsters was carried out in accordance with the method described by Bhatnagar *et al.* (1989). Five to six animals were used for each test sample. Same numbers of hamsters were kept as untreated controls. Treatment of miltefosine and paromomycin was given for 5 days (after establishment of appropriate infection) by oral (po) and intra peritoneal (ip) routes respectively. Picroliv was administered orally for 12 days. To assess the effect of drugs, spleen biopsy was performed on each animal after 28 days of last drug

administration. Intensity of infection in both, treated and untreated animals, as also the initial count in treated animals is compared and the efficacy is expressed in terms of percentage inhibition (PI) using the following formula:-

PI = 100- [ANAT x 100/ (INAT x TIUC)]

Where PI is Percent Inhibition of amastigotes multiplication, ANAT is Actual Number of Amastigotes in Treated animals, INAT is Initial Number of Amastigotes in Treated animals and TIUC is Times Increase of parasites in Untreated Control animals.



1.4.2.1. Evaluation of individual drug responses

Initially dose optimization of miltefosine, paromomycin and picroliv was done. Miltefosine was evaluated at doses ranging from 40 mg/kg to 2.5 mg/kg for 5 days. A dose of 5 mg/kg was selected as the sub-curative dose for combination studies. Paromomycin was tested at doses ranging from 100 mg/kg to 12.5 mg/kg for 5 days and 80 mg/kg was found appropriate for combination therapy. Picroliv was given at doses ranging from 20 mg/kg to 5 mg/kg for 12 days and 10 mg/kg dose was selected for further experiments.

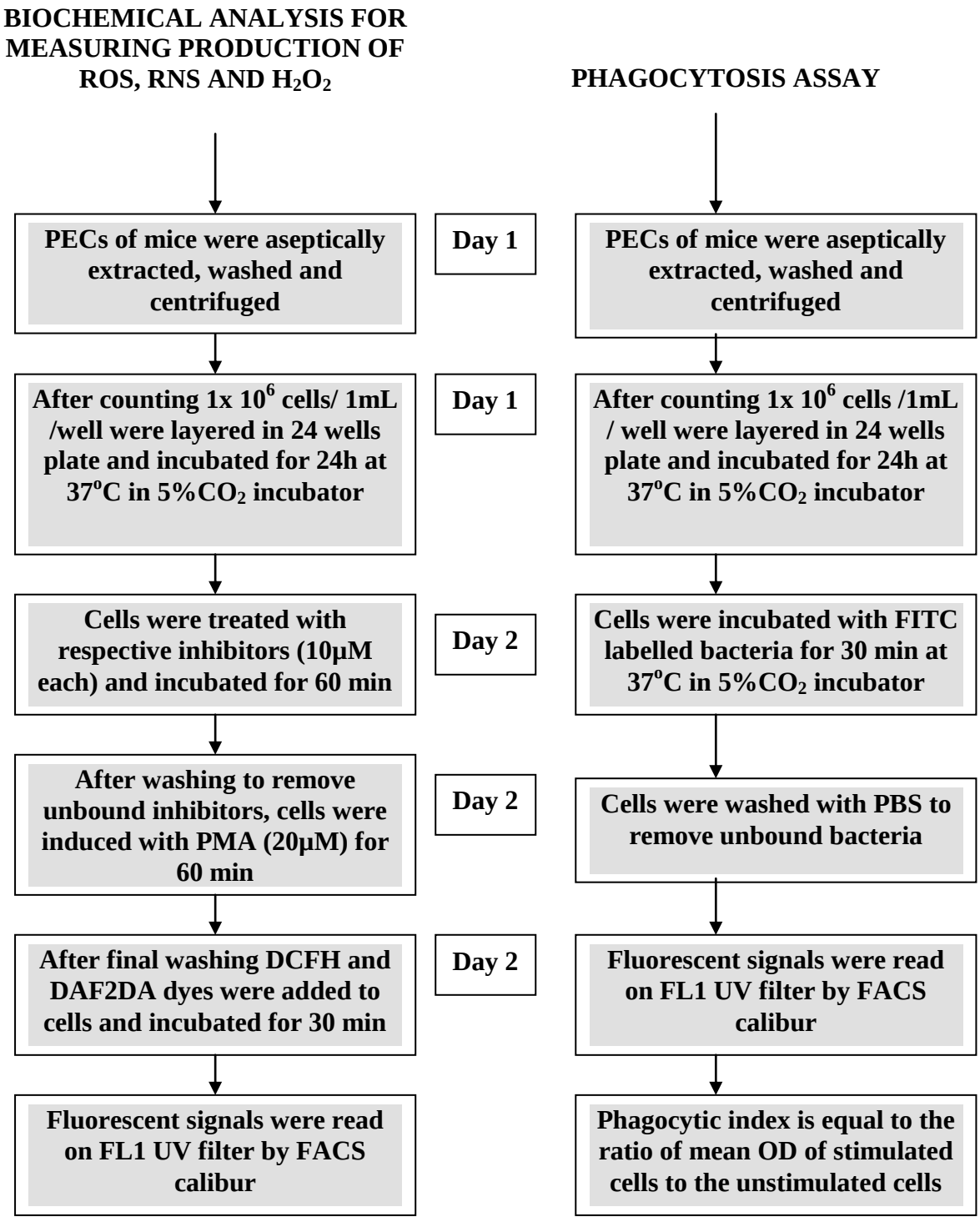
1.4.2.2. Evaluation of combination therapy

For evaluation of drug combination six groups of hamsters, each consisting of five-six animals in two replicates was used. Animals of Group I received picroliv (10 mg/kg for 12 days, po), Group II received paromomycin (80 mg/kg for 5 days, ip), Group III received miltefosine (5 mg/kg for 5 days, po), Group IV received paromomycin + miltefosine, Group V received paromomycin + miltefosine + picroliv, Group VI received paromomycin + Picroliv, Group VII received miltefosine + picroliv and Group VIII received deionized water and served as controls for all the groups. The percent inhibition was calculated for all drug-treated groups in relation to a non treated group.

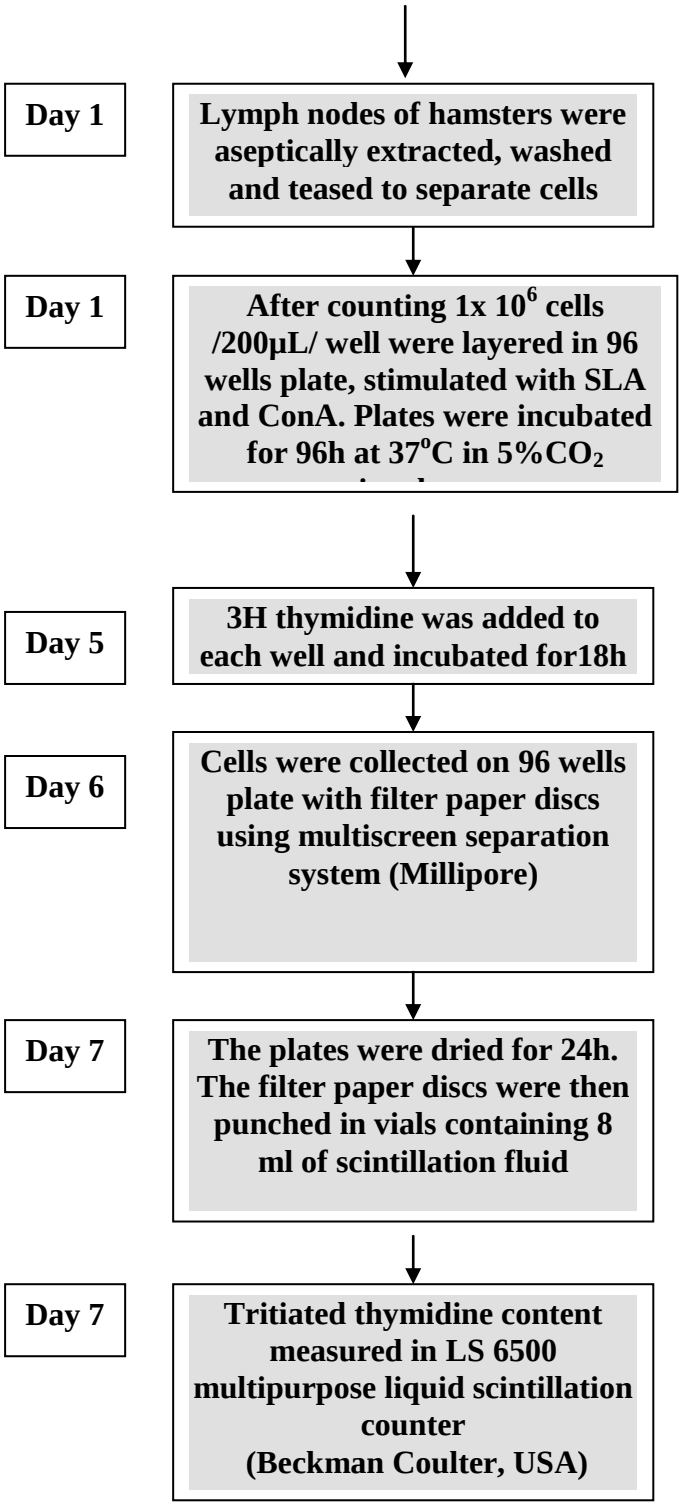
1.4.2.3. Immunological & Biochemical Analysis

- Reactive oxygen species production assay using inhibitor pentoxifylline (PTx).
- Hydrogen peroxide production assay using inhibitor sodium azide (NaN_3).
- Reactive Nitrogen species production assay using inhibitor N-nitro-L-arginine methyl ester (L-NAME).
- Phagocytosis assay.
- Lymphocyte proliferation assay

Immunological & Biochemical Analysis in *L.donovani*/hamster model



LYMPHOCYTE PROLIFERATION ASSAY



1.5. Statistical Analysis

Results are presented as mean ± S.D. of two experiments and analysis of data is carried out by Bonferroni's multiple comparison tests and Dunnett's multiple comparison tests. Differences with $P< 0.05$ were considered significant. Sub-curative dose was determined by probit analysis (Finney, 1971).

2. RESULTS

2.1. Apoptotic-Necrotic profiling to determine phosphatidylserine exposure in promastigotes

Dot plots of apoptotic-necrotic profiling have been presented in Fig.1. It was observed that untreated promastigotes were Annexin V FITC and PI negative, while in case of paromomycin treated sample 82% cells were in late apoptotic phase (Annexin V FITC and PI positive). In miltefosine treated sample 60% cells were in early apoptotic phase (Annexin V FITC positive and PI negative) and 26% in late apoptotic phase (Annexin V FITC and PI positive). Picroliv *per se* has shown insignificant cell death comparable with untreated control. It was observed that promastigotes when exposed to picroliv in combination with either miltefosine or paromomycin, percentage of cells undergoing apoptosis decreases. Combination of paromomycin and miltefosine caused 91% cell death but when picroliv is added to this combination cell death decreases to 88%. Such an observation supports antioxidative nature of picroliv by the virtue of which it prevents cells from apoptotic damage.

- Lower right [LR]- Annex FITC +ve cells - Early apoptotic stage
- Upper right [UR]- Annex FITC+ve and PI +ve - Late Apoptotic stage
- Lower left [LL]- Unstained viable cells
- Upper left [UL]- PI +ve cells - In necrotic stage

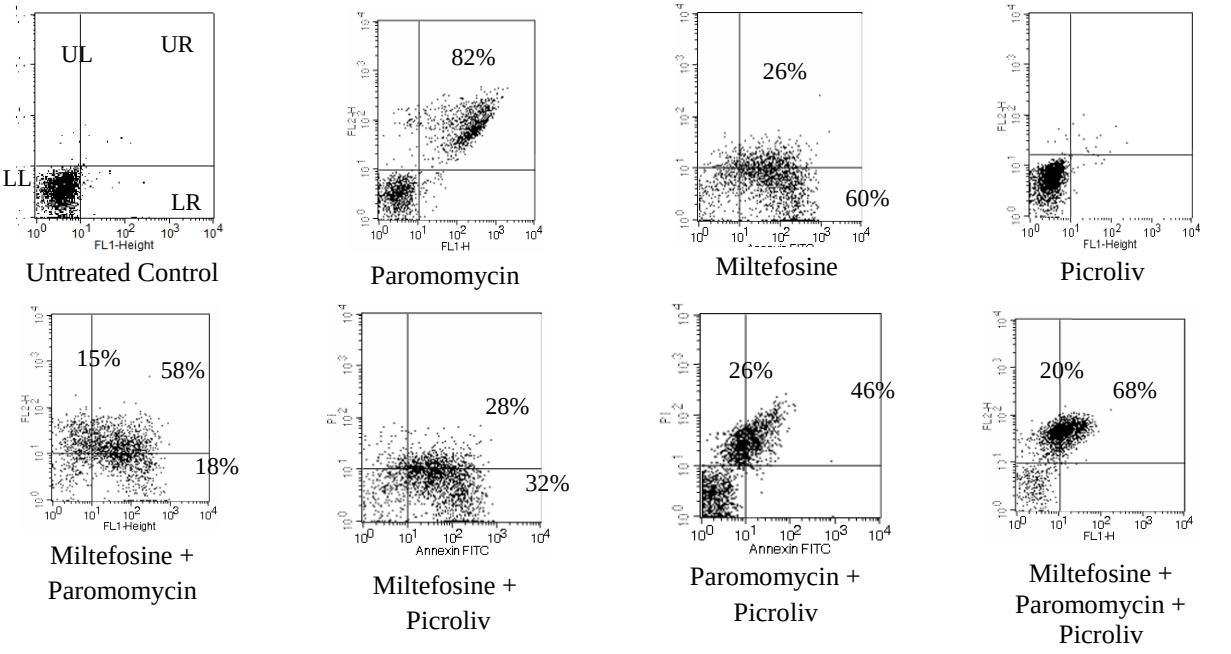


Fig.1 Phosphatidylserine exposure in promastigotes

Legend.1 *L.donovani* promastigotes ($2x10^6$) treated with various drug combinations for 72h at 24 ± 2^0C. For measuring phosphatidylserine exposure, treated and untreated promastiogotes washed in PBS by centrifugation followed by addition of Annexin V- FITC and PI to each tube and incubated for 30 min at room temperature. Samples were read on FACS Calibur. Positioning of quadrants on Annexin V/ PI dot plots was performed and living cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI), late apoptotic cells (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+) were distinguished.

2.2. Measurement of mitochondrial transmembrane potential in promastigotes.

Drop in mitochondrial membrane potential was measured after a period of 72h by staining with JC-1 dye. Drop in potential of treated promastigotes is compared with untreated control. Drop in potential observed was 69%, 60%, 67% and 55% in case of miltefosine, paromomycin, miltefosine + picroliv, paromomycin + picroliv treated promastigotes respectively. A sharp mitochondrial membrane hypo-polarization was observed in

promastigotes treated with miltefosine + paromomycin (95%) and it was almost equal to the drop caused by combination of paromomycin, miltefosine and picroliv (96%). No marked difference was observed in drop due to picroliv either alone or in combination with miltefosine and paromomycin (Fig.2).

- Upper right [UR]: Red Fluorescent cells- J-aggregate-No drop in mitochondrial membrane potential.
- Lowe right [LR]: Green fluorescent cells – J-monomer- Drop in mitochondrial membrane potential

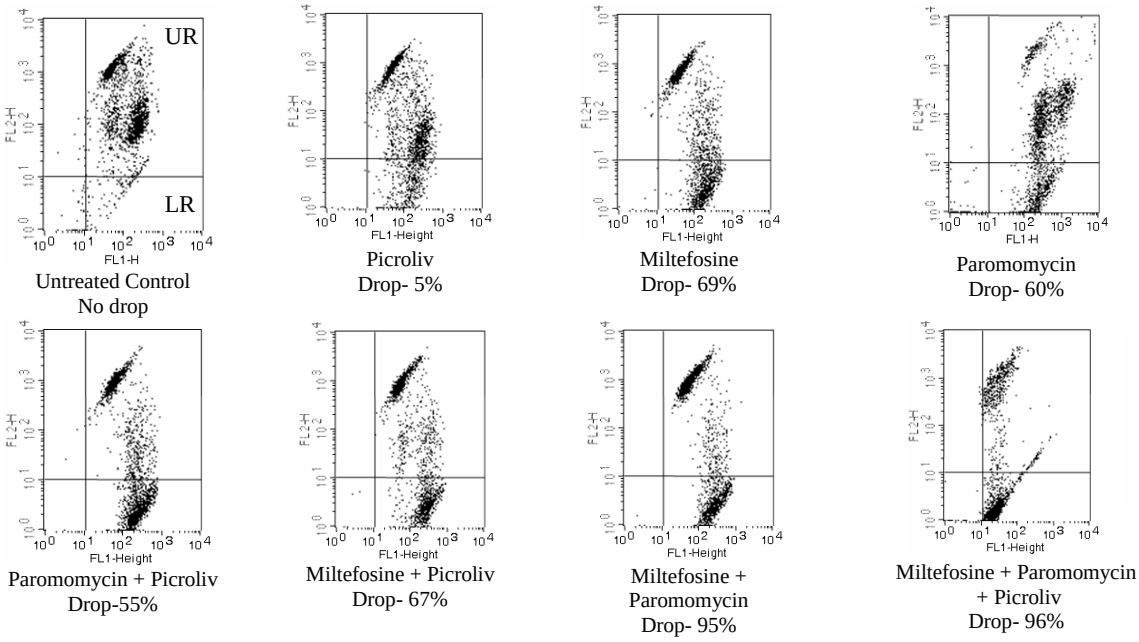


Fig.2 Drop in mitochondrial membrane potential

Legend.2. *L.donovani* promastigotes ($2x10^6$) treated with various drug combinations for 72h at 24 ± 2^0C incubator. At 72h Drug-treated and control promastigotes were washed with PBS by centrifugation (3100 rpm/10min.) and then stained with JC-1 (3 mM in PBS) and analysed by flow cytometry. JC-1 aggregates within mitochondria and fluoresce red at higher transmembrane potentials while at lower transmembrane potentials, JC-1 cannot accumulate within the mitochondria and instead remains in the cytosol as monomers, which fluoresce green.

2.3. *In vitro* drug responses against intra-macrophagic amastigotes

Results of antiamastigote efficacy of individual drugs and their various combinations have been presented in Fig.3. Combination of miltefosine + paromomycin (80.2%) has shown significant antileishmanial efficacy (P<0.01) as compared to individual miltefosine (53.6%)

and paromomycin (61.8%) treated groups. Picroliv alone showed no significant antileishmanial efficacy (13%) but when it was given in combination with miltefosine (60%) and paromomycin (72.2%) individually, enhanced efficacies were observed. Combination of all the three drugs has given antileishmanial efficacy of 95% which was highly significant (P<0.001) compared to other combinations as well as individual drugs.

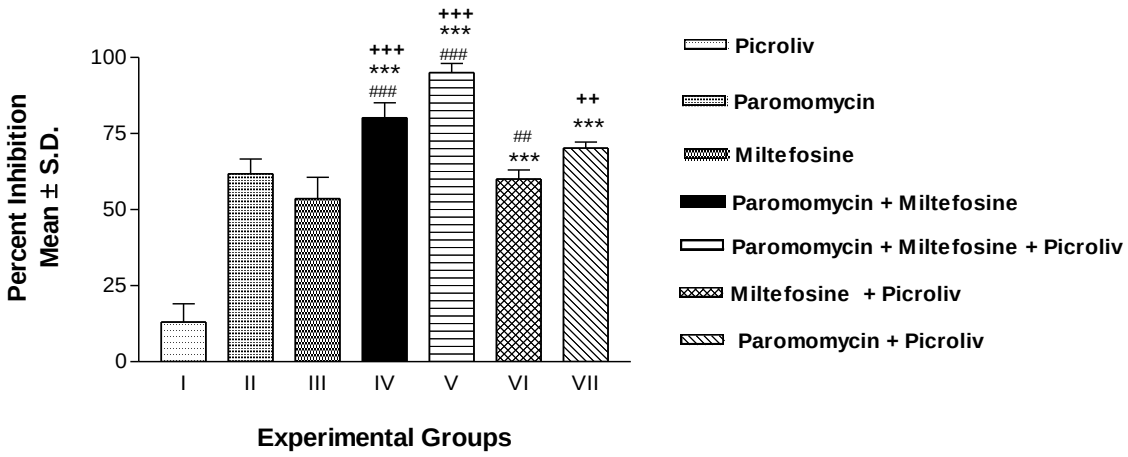


Fig.3 *In vitro* efficacy evaluation of drugs alone and in combinations

Legend.3 1x10^6 PECs of BALB/c mice infected with amastigotes at a ratio of 1:7 in 16 well chamber slides (NUNC). Drug treatment was given for 72h and then slides were stained with Geimsa stain. Percent Inhibition (P.I.) ± S.D. was calculated by comparing amastigote count of treated wells to untreated ones. Significance among different groups was calculated by Boneferroni's multiple comparison test (I vs V – P<0.001; I vs VI- P<0.01; I vs VII- P<0.001***), (II vs IV- P<0.001; II vs V- P<0.001; II vs VII- P<0.01$^{###}$), (III vs IV- P<0.001; III vs V- P<0.001; III vs VI- P<0.01^{+++}).

2.4. Evaluation of individual drugs and their various combinations in *L.donovani*/hamster model

2.4.1. Dose optimization of miltefosine

Miltefosine was tested at various doses ranging from 40 mg/kg to 2.5 mg/kg for five days by po route. Parasite inhibition observed at 40 mg/kg was 99% followed by 92%, 80%, 50% and 34.5% at 20, 10, 5, 2.5 mg/kg doses respectively. Best dose for combination trial was selected as 5 mg/kg as it was sub-curative and non toxic (Fig.4).

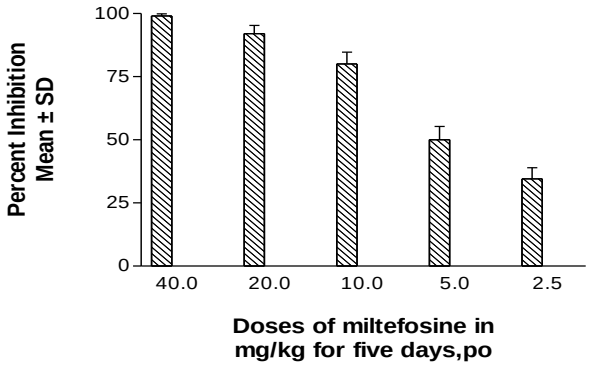


Fig.4 Dose optimization of miltefosine

Legend.4 *L.donovani* infection was given to hamster intracardically ($1x10^7$amastigotes/animal). Pre treatment biopsy was done to examine parasitic burden on D_{15} post infection. Animals with appropriate parasitic counts were treated with miltefosine at different dosages for five days by oral route. Post treatment biopsy was done on D_{28} of treatment and mean percent inhibition (P.I.) ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.4.2. Dose optimization of paromomycin

Paromomycin was evaluated at doses ranging from 100 mg/kg to 20 mg/kg for five days by ip route. Parasite inhibition observed at 100 mg/kg was 84% followed by 72.1%, 46.7% and 22.5% at 80, 40 and 20 mg/kg doses respectively. Dose of 80 mg/kg was selected for combination trial as it showed consistent efficacy up to day 28 post treatment and was least toxic (Fig.5).

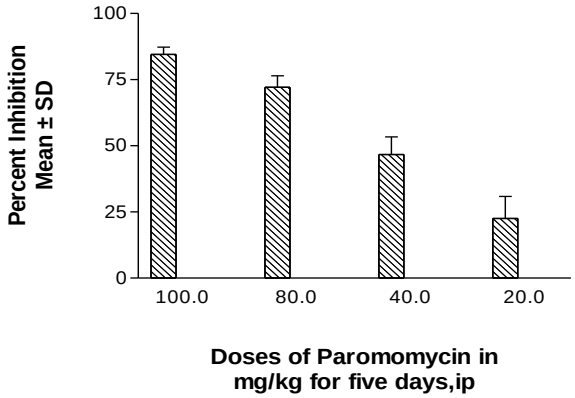


Fig.5 Dose optimization of paromomycin

Legend.5 *L. donovani* infection was given to hamster intracardically ($1x10^7$amastigotes/animal). Pre treatment biopsy was done to examine parasitic burden on D_{15} post infection. Animals with appropriate parasitic counts were treated with paromomycin at different dosages for five days by intraperitoneal route. Post treatment biopsy was done on D_{28} of treatment and mean percent inhibition (P.I.) ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.4.3. Dose optimization of picroliv

Picroliv was optimized at different doses ranging from 5 mg/kg to 40 mg/kg but it didn't exhibit any dose dependent antileishmanial efficacy (18-22% parasite inhibition). Based on earlier findings from our lab, 10 mg/kg for 12 days dose by po route was used in this study (Fig.6).

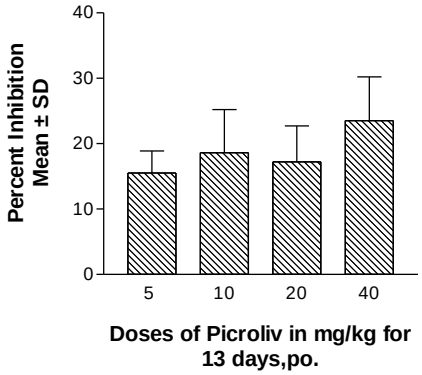


Fig.6 Dose optimization of picroliv

Legend.6 *L. donovani* infection was given to hamster intracardically ($1x10^7$amastigotes/animal). Pre treatment biopsy was done to examine parasitic burden on D_{15} post infection. Animals with appropriate parasitic counts were treated with picroliv at different dosages for thirteen days by oral route. Post treatment biopsy was done on D_{28} of treatment and mean percent inhibition (P.I.) ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

2.4.4. Combination therapy of miltefosine, paromomycin and picroliv

The data of the efficacy of individual drugs and their combinations have been displayed in Fig.7. Paromomycin and miltefosine separately showed antileishmanial efficacy of 72.5% and 48.8% respectively. Picroliv *per se* exhibited nominal antileishmanial activity. However,

when picroliv was given with miltefosine, its efficacy was significantly (P<0.001) enhanced from 48.8% to 72.5%. Efficacy of miltefosine has also been enhanced when administered in combination with paromomycin from 48.8% to 84.5% (P<0.001). A marked increase in antileishmanial efficacy was observed when picroliv was given with paromomycin (P<0.01) as compared with *per se* paromomycin (71.9% to 87.0%). In group of hamsters receiving combination of all the three drugs namely paromomycin, miltefosine and picroliv, percent inhibition was recorded as 96.6% which was significantly (P<0.001) higher than the efficacies of individual drugs paromomycin (72%), miltefosine (48.8%) & picroliv (17.5%).

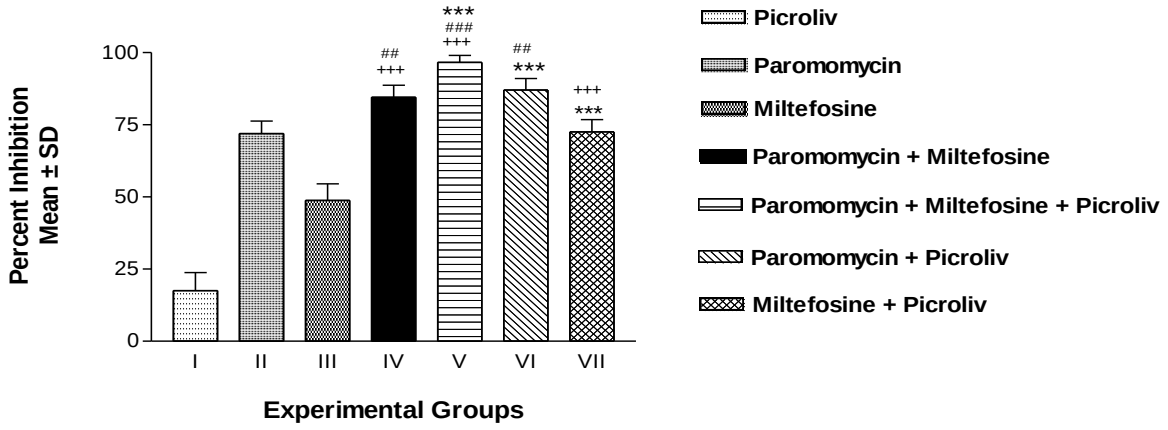


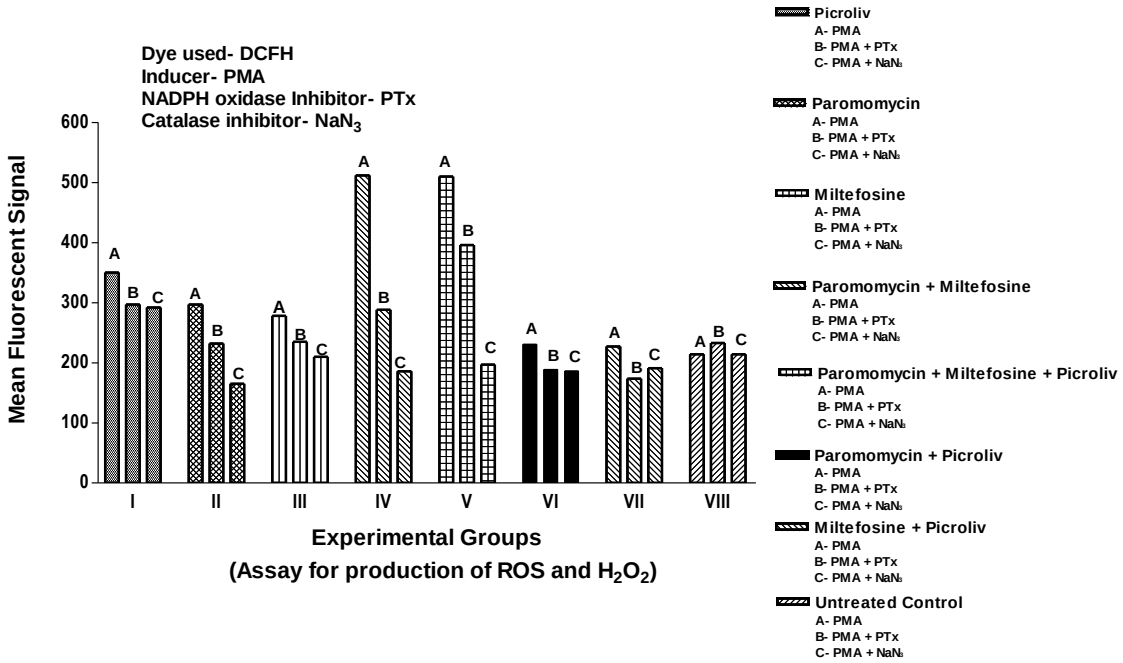
Fig.7 Combination therapy of paromomycin, miltefosine and picroliv

Legend.7 *L. donovani* infection was given to hamster intracardically (1x10^7amastigotes/animal). Pre treatment biopsy was done to examine parasitic burden on D_{15} post infection. Animals with appropriate parasitic counts were treated with different drugs alone as well as in combinations (adjunct). Post treatment biopsy was done on D_{28} of treatment and mean percent inhibition (P.I.) ± S.D. was calculated by comparing parasitic burden of treated groups to control animals. Significance among different groups was calculated by Boneferroni's multiple comparison test (I vs V – P<0.001; I vs VI- P<0.001; I vs VII- P<0.001^{***}); (II vs IV- P<0.01; II vs V- P<0.001; II vs VI- P<$0.01^{\#\#\#}$); (III vs IV- P<0.001; III vs V- P<0.001; III vs VII- P<0.001^{+++}).

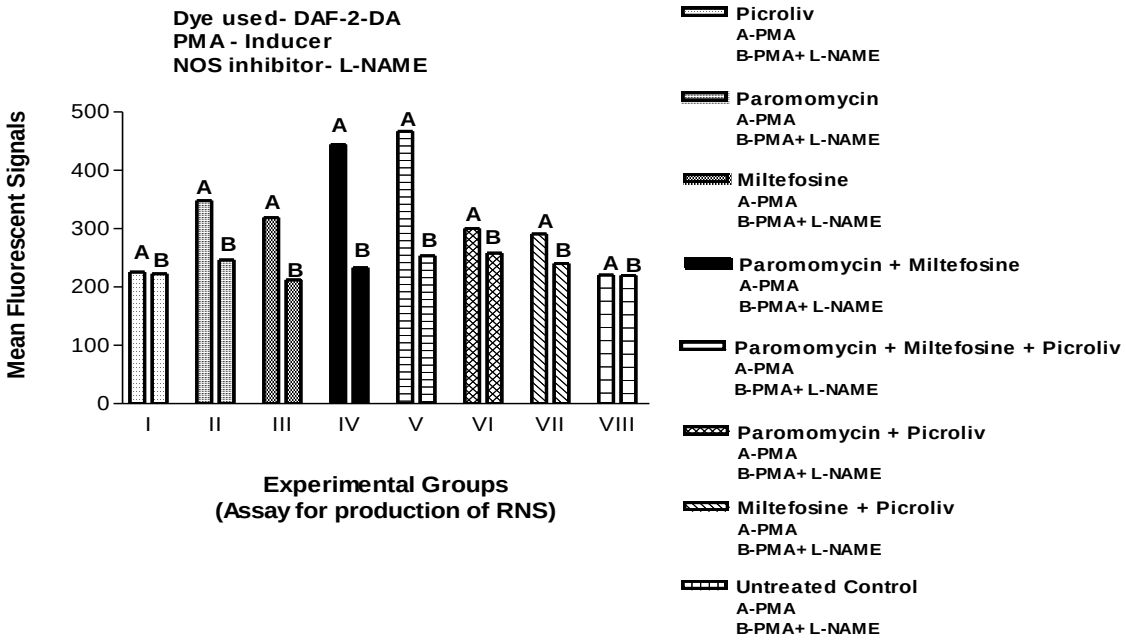
2.5. Alterations in biochemical parameters

Production of toxic oxygen and nitrogen metabolites was measured by FACS in which ROS production in animals treated with paromomycin and miltefosine separately was better as

compared to untreated control (P<0.01, P<0.01 respectively).Animals dosed with combination of paromomycin + miltefosine have also shown significant ROS, H_2O_2 and RNS production (P<0.001). Similar level of toxic metabolites production (P<0.001) was observed in animals treated with combination of all the three drugs. Picroliv alone did not exhibit any toxic oxygen metabolite production (Fig.8).



A) Production of ROS and H_2O_2.



B) Production of RNS.

Fig.8. Alterations in Biochemical parameters

Legend.8 Peritoneal exudates of animals from different treatment groups were isolated by sacrificing the animal. Production of ROS, RNS and H_2O_2 on PMA induction was studied by FACS analysis. Fluorescence obtained in presence and absence of respective inhibitors was then compared. Significance of activity of different treated groups at D_{28} post treatment was assessed against untreated (control) animals by Dunnett's multiple comparison tests. **For ROS** : (VIII vs I – $P<0.05$; VIII vs II – $P<0.01$; VIII vs III – $P<0.05$; VIII vs VI– $P<0.001$; VIII vs V – $P<0.001$; VIII vs VI – $P<0.05$; VIII vs VII – $P<0.01$). **For H_2O_2**: (VIII vs I – $P<0.05$; VIII vs II – $P<0.01$; VIII vs III – $P<0.01$; VIII vs IV– $P<0.001$; VIII vs V – $P<0.001$; VIII vs VI – $P<0.01$; VIII vs VII – $P<0.05$). **For RNS**: (VIII vs I – ns; VIII vs II – $P<0.01$; VIII vs III– $P<0.01$; VIII vs VI– $P<0.001$; VIII vs V – $P<0.001$; VIII vs VI – $P<0.01$; VIII vs VII – $P<0.01$).

2.6. Phagocytosis assay

From the results presented in Fig.9 it was observed that picroliv *per se* showed nominal increase in phagocytic index (PI: 0.43 ± 0.02) as compared to untreated control group (PI: 0.38 ± 0.03). Picroliv when given in combination with paromomycin (PI: 2.41 ± 0.05) or miltefosine (PI: 1.97 ± 0.05) it has significantly enhanced their phagocytic activity ($P<0.001$,

P<0.001 respectively). Paromomycin (PI: 1.94 ± 0.04) and miltefosine (PI: 1.05 ± 0.06) *per se* and in combination (PI: 2.98 ± 0.05) has also shown high phagocytic responses (*P*<0.001, *P*<0.01 and *P*<0.001 respectively). Animals treated with combination of all the three drugs have shown maximum phagocytic ability (PI: 3.55 ± 0.03) as compared to untreated control group (*P*<0.001).

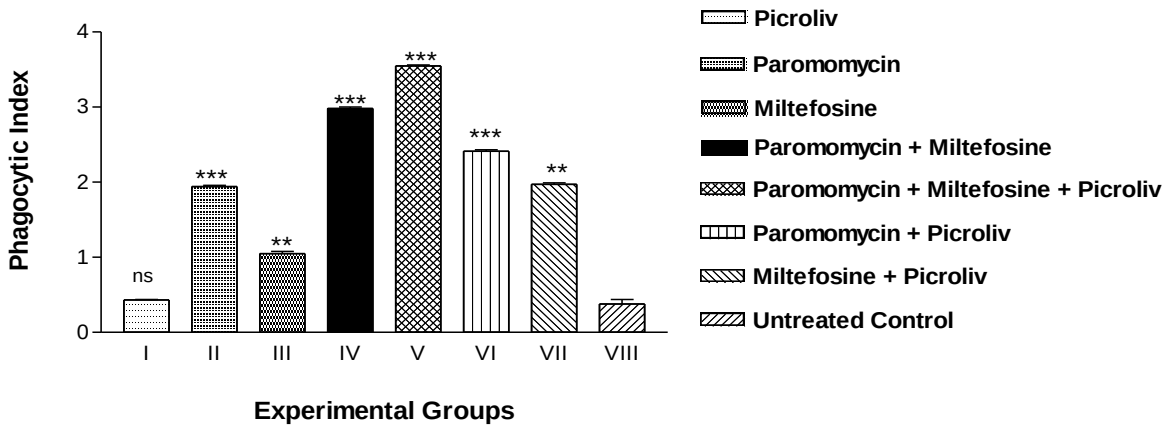


Fig.9 Phagocytic responses

Legend.9 Peritoneal exudates of animals from different treatment groups were isolated by sacrificing the animals. Macrophages stimulated with FITC labeled bacteria and incubated. Fluorescence of stimulated and unstimulated cells of each group compared and significance of activity of different treated groups on D_{28} post treatment was assessed against untreated (control) animals by Dunnett's multiple comparison tests (VIII vs I- ns; VIII vs II – *P*< 0.001; VIII vs III – *P*< 0.01; VIII vs IV - *P*< 0.001; VIII vs V- *P*< 0.001; VIII vs VI – *P*<0.001; VIII vs VII – *P*< 0.01***).

2.7. Lymphocyte proliferation response

Results have been displayed in Fig.10. As compared to untreated control (SI: 0.72 ± 0.05), animals treated with paromomycin (SI: 1.6), miltefosine (SI: 1.23 ± 0.02) and picroliv (SI: 1.37 ± 0.07) individually showed significant (*P*<0.001, *P*<0.01, *P*<0.01 respectively) proliferative response to specific mitogen (SLA). Animals treated with paromomycin + miltefosine (SI: 2.0 ± 0.05), miltefosine + picroliv (SI: 1.71 ± 0.03), paromomycin + picroliv (SI: 1.8 ± 0.05) and all the three drugs together (SI: 3.13 ± 0.01) have also shown significant

proliferative responses (P<0.001, P<0.001, P<0.001, P<0.001) as compared to untreated control group.

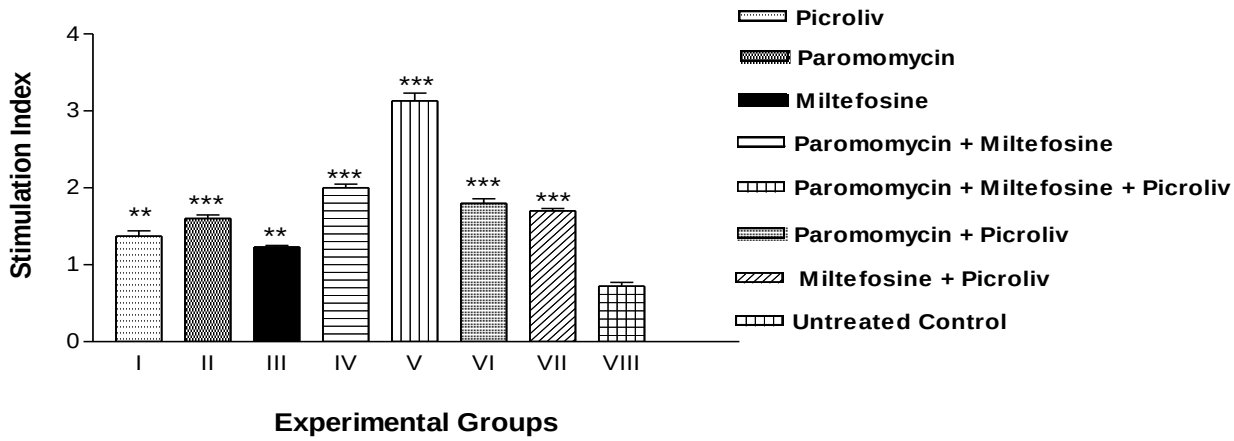


Fig.10 Lymphocyte proliferation responses

Legend.10 Mesenteric Lymph nodes of animals from different treatment groups were isolated by sacrificing the animal. Lymphocytes were induced with specific SLA. Extent of proliferation was correlated to degree of H_3(thymidine) incorporation in DNA of newly formed cells .Proliferation were read on PRIAS CLD / 400 liquid scintillation counter and stimulation index was calculated. Significance of activity of different treated groups on D_{28} post treatment was assessed against untreated (control) animals by Dunnett's multiple comparison test (VIII vs I- P<0.01; VIII vs II – P< 0.001; VIII vs III – P< 0.01; VIII vs IV - P< 0.001; VIII vs V- P< 0.001; VIII vs VI- P< 0.001; VIII vs VII- P< 0.001***).

3. DISCUSSION

Several successful reports on the combination of chemotherapy with immunotherapy for treatment of leishmaniasis have been published so far. Solgi *et al.* (2006) showed effective combination of immunomodulator thalidomide with glucantime in murine VL. Adjunct therapy of muramyl peptide with stibanate against VL in hamsters was also found quite effective (Puri *et al.*, 2005). Immunomodulator imiquimod in combination with paromomycin effectively treated cutaneous leishmaniasis caused by *L. major* (El-On J *et al.*, 2007). Based on these findings and previous work in our lab (Gupta *et al.*, 2004 and 2005), we have used

immunomodulator, picroliv in combination with standard antileishmanial drugs miltefosine and paromomycin. Curative dose of both these drugs have their own side effects viz. prominent teratogenicity by miltefosine and less prominent ototoxicity /nephrotoxicity by paromomycin (Chappuis *et al.,* 2007). In order to lower the chances of toxicity, we have reduced the dose of miltefosine and paromomycin in our study. Study was initiated with dose optimization of individual drugs and best effective (paromomycin) and sub-curative (miltefosine) dose was selected for combination. Results of the present study indicate that picroliv significantly enhanced the antileishmanial efficacy of combination of miltefosine plus paromomycin. Leishmanicidal activity of the combination was maintained up to 28th day post treatment. This combination also exhibited significant phagocytic and lymphocyte proliferation responses. Picroliv when given with miltefosine and paromomycin individually, moderate increase in efficacy from 48.8% to 72% and from 84.5% to 87% respectively was witnessed. Significant generation of ROS, RNS and H_2O_2 was observed in animals treated with miltefosine and paromomycin, however, addition of picroliv to this combination did not alter the level of metabolites. Reason for such behaviour of picroliv can be conferred to its free radical scavenging (Ray *et al.,* 2002) and antioxidative (Seth *et al.,* 2003) property by virtue of which it prevents production of excess toxic metabolites. The anti cell damaging effect of picroliv was also reflected by apoptosis study, where it has mildly reduced the percentage of cells undergoing apoptosis due to combined action of miltefosine and paromomycin. These results corroborate the earlier findings of Puri *et al.* (1992); Ray *et al.* (2002) and Pathak and Khandelwal, 2008. Picroliv possesses dual activity, at one hand it is an immuno-stimulator while on the other hand it is a hepato-protector (Puri *et al.,* 1992; Dwivedi *et al.,* 1992; Chander *et al.,* 1994). Picroliv is under Phase III trial and is expected to be in market very soon for human use. Findings of this study suggested that picroliv helped antileishmanial drugs in enhancement of parasite inhibition at their less toxic doses. Since,

VL is associated with immunosuppression, the application of picroliv in combination of paromomycin and miltefosine appears to be a good strategy for treatment.

SUMMARY

Visceral Leishmaniasis (VL), also known as kala-azar in the Indian sub-continent, is caused by the protozoan parasites *Leishmania donovani* and *Leishmania infantum* (*Leishmania chagasii*), and is a potentially fatal disease with a worldwide distribution, in Asia, East Africa, South America and the Mediterranean region. The parasites are transmitted through the bite of female phlebotomine sand flies and in the human host are obligate intracellular parasites of the reticuloendothelial system, surviving and multiplying in different macrophage populations. In patients who develop symptoms, presentation is insidious with development of splenomegaly, irregular fevers, anaemia, pancytopaenia, weight loss and weakness occurring progressively over a period of weeks or even months. Almost all clinically symptomatic (non-immune) patients die within months if untreated.

VL persists today in poor, remote, and in certain politically unstable areas, where there is limited health care and patients have little access to affordable medications. Almost half of clinical cases occur in children. It is estimated that about 90% of the 500,000 new cases arising each year occur in the rural areas of South Asian countries (India, Nepal, Bangladesh), Sudan, Ethiopia and Brazil and only 30% of cases are reported.

Chemotherapy remains the most important element in the control of anthroponotic visceral leishmaniasis. The first-line therapy included sodium stibogluconate (SbV) which has unfortunately developed resistance in some areas of Bihar where failure rates of up to 65% have been reported and the use of antimony has been abandoned. Pentamidine also went out of favour for treatment of VL. The use of liposomal amphotericin-B is limited due to high market price. A major milestone in chemotherapy of VL was the discovery of miltefosine, an analogue of phosphatidylcholine initially developed as an anticancer agent. It is an effective oral drug but its use in women of child-bearing age is restricted due to teratogenicity. In addition, it has a long half-life, which might encourage the emergence of resistance once its

use becomes widespread. Another promising drug added to antileishmanial chemotherapy was paromomycin (formerly known as aminosidine). It was registered in India in August, 2007 for treatment of VL. Advantages of paromomycin include fewer side effects, activity against a wide variety of pathogens and its low cost (US$5–10 per treatment). Currently, the non-profit group Drugs for Neglected Diseases Initiative and the Institute for One World Health is conducting studies on paromomycin (as monotherapy and in combination) against VL in Africa and Asia. Unfortunately, all of these drugs have significant drawbacks that limit their utilization in disease endemic areas. These include route of administration, length of treatment (21 to 28 days), toxicity and cost.

Combination therapy is the cornerstone of our VL strategy. It offers the following important advantages:

1. Shorter course of treatment.

2. Potentially this leads to better tolerability and lower cost of treatment. Also importantly, this reduces the workload on the health systems in resource-limited areas.

3. Most importantly, embedded in this strategy is the potential to reduce the risk of resistance development and to prolong the life span of present drugs.

Cure of leishmaniasis appeared to be dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen metabolites to kill the intracellular amastigotes. Several studies have been reported which showed use of endogenous biologicals, microbial derivatives or synthetic compounds as immunomodulators in both animals and humans. Amongst them most frequently used immunomodulators were BCG (Bacille Calmette–Guèrin), MDP (muramyl dipeptide), trehalose mycolate, glucan, tuftsin, and protein-A which have a direct effect on macrophages. Previous reports showed that combination of biological immunomodulators such as IFN-γ (interferon- gamma), imiquimod and a hexapeptide (analogous to human beta-casein

fragment) to antileishmanial drug (antimonials) have enhanced the leishmanicidal activity in the treatment of VL. Based on these findings the present work was designed to evaluate the effect of immunomodulators namely CpG oligodeoxynucleotides (ODN) and picroliv in combination with standard antileishmanial drugs (miltefosine and paromomycin) in *L.donovani*/mice and hamster model.

Combination therapy using CpG ODN (1826, 2006) with miltefosine

CpG ODNs are a class of pharmacotherapeutic agents, characterized by the presence of an unmethylated CG dinucleotide in specific base sequence contexts (CpG motif). These CpG motifs are not seen in eukaryotic DNA, but are present in bacterial DNA to which they confer immunostimulatory properties. These properties include induction of a Th1-type response with prominent release of IFN-α, IL-12, and IL-18. They (18–24 bp in length) possess immunomodulatory characteristics similar to bacterial DNA. CpG ODN has demonstrated substantial potential as vaccine adjuvants, and as mono- or combination therapies for the treatment of cancer and infectious and allergic diseases. They have also shown effect in multiple rodent and primate models of asthma and other allergic diseases, with encouraging results in some early human clinical trials. Applicability of CpG ODN has also been exploited in the field of leishmaniasis especially as immunomodulator and adjuvant with various immunogens. In this study attempts have been made to evaluate immunomodulatory effect of unmethylated CpG on the efficacy of miltefosine, at a substantive dose against experimental visceral leishmaniasis in rodent models.

Adjunct effect of two fully thioated class B CpG ODNs (1826, 2006) in free and liposomal form on the efficacy of miltefosine was explored using *L.donovani* / BALB/c mouse and hamster model. Results clearly indicate that liposomal encapsulation enhanced antileishmanial efficacy of free CpG ODN (1826, 2006). Co-administered liposomal CpG ODN (1826, 2006) and miltefosine showed a better inhibitory effect than free CpG ODN +

miltefosine, free CpG ODN alone or miltefosine alone. Empty liposome preparation did not exhibit any antileishmanial efficacy. Results of immunological assays in mice suggested that CpG ODN (1826) potentiated the cell mediated immunity, evident from significant rise in Th1 cytokines and down regulation of Th2 cytokines. Liposomal encapsulation of CpG ODN (1826) enhanced IL-2, TNF and IFNγ levels. In biochemical assay, liposomal CpG ODN (1826) combined with miltefosine resulted in remarkable production of NO, ROS and H_2O_2. Thus, the promising antileishmanial efficacy of liposomal CpG ODN (1826) + miltefosine is strongly supported by enhancement of Th1 cytokines as well as NO, ROS and H_2O_2 levels. CpG ODN 2006 was evaluated with sub-curative miltefosine and it was observed that its efficacy profile was in correlation with efficacy of CpG ODN 1826. To further strengthen the study, CpG ODN 2006 was also evaluated with slightly higher sub-curative doses of miltefosine namely at 5 mg/kg and 10mg/kg for five days in mice and hamsters respectively. This combination significantly enhanced the efficacy of miltefosine in both the rodent models compared to the combination using sub-curative dose of miltefosine. The efficacy of this combination was comparable with the efficacy of the curative dose of the miltefosine. Conclusively this study offers the promise of this combination (liposomal CpG ODN with miltefosine) as a novel therapeutic approach for a safer treatment of VL using a significantly reduced dose of miltefosine.

Combination therapy using picroliv with paromomycin and miltefosine

Picroliv, standardized fraction from the alcoholic extract of root and rhizome of *P. kurroa*, is a mixture of iridoid glycosides containing 55–60% of picroside-I and kutkoside in a ratio of 1:1.5. It has been found to stimulate both humoral & cell mediated immune responses. Its major medicinal properties reported in modern literature are antioxidant, free radical scavenging, immunomodulatory, hepatoprotective, testicular protective, cholagogue and anticholestatic. Some other properties which contribute to its usefulness are anti-

inflammatory, cardioprotective and moderate antiviral activity. It was also found to enhance antileishmanial efficacy of miltefosine when given in combination and reduce the side effects of multi drug therapy in TB treatment.

This study was initiated with dose optimization of individual drugs and best effective (paromomycin) and sub curative (miltefosine) dose was selected for combination. Results indicated that picroliv significantly enhanced the antileishmanial efficacy of combination of miltefosine + paromomycin. Leishmanicidal activity of the combination was maintained up to 28th day post treatment. This combination also exhibited significant phagocytic and lymphocyte proliferation responses. Picroliv when given with miltefosine and paromomycin individually, moderate increase in efficacy was witnessed. Significant generation of ROS, RNS and H_2O_2 was observed in animals treated with miltefosine and paromomycin, however, addition of picroliv to this combination did not alter the level of toxic metabolites. Reason for such behaviour of picroliv can be conferred to its free radical scavenging and antioxidative property by virtue of which it prevents production of excess toxic metabolites. Findings of this study suggested that picroliv helped antileishmanial drugs in enhancement of parasite inhibition at their less toxic doses.

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List of Publications

➢ Research Papers

1. **Shraddha A Sane**, Nishi, W. Haq and S. Gupta. CpG oligodeoxynucleotides (ODN) augmented antileishmanial activity of miltefosine in combination against experimental Visceral Leishmaniasis. *Journal of antimicrobial chemotherapy*.2010; 65(7):1448-1454.
2. **Shraddha A Sane**, Nishi and S. Gupta. *Leishmania donovani*: Immunomodulatory effect of picroliv on the efficacy of paromomycin and miltefosine in experimental visceral Leishmaniasis. *Experimantal Parasitology*. 2010.doi:10.1016/j.exppara.2010.09.003
3. Nishi, **Shraddha A Sane** and S. Gupta. Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator-picroliv. *Parasitology Research*. Manuscript ID PR-2010-0414 (SY-05) (In press).
4. A. Verma, S. Srivastava, **S. A. Sane,** V.K. Marrapu, N. Srinivas, M. Yadav, K. Bhandari, and S. Gupta. Antileishmanial activity of benzocycloalkyl azole oximino ethers: The conformationally constraint analogues of oxiconazole. *Acta Tropica*. 2010.doi: 10.1016/j.actatropica.2010.10.011.
5. N. Shakya, **S. A. Sane**, P. Vishwakarma, P. Bajpai and S. Gupta. Improved treatment for Visceral Leishmaniasis (Kala-azar) by using combination of ketoconazole and miltefosine with an immunomodulator-picroliv. Parasitology. MS ID PAR-2010-0317.
6. N. Shakya, **S. A. Sane**, P. Vishwakarma, and S. Gupta 'Antileishmanial Efficacy of Fluconazole and Miltefosine in Combination with an Immunomodulat or Picroliv', *Medicinal Chemistry Research.* 19, S96- S96, 2010.
7. V.P. Pandey, S.S. Bisht, M. Mishra, A. Kumar, M.I. Siddiqi, A. Verma, M. Mittal, **S. A. Sane**, S. Gupta, R.P. Tripathi. Synthesis and molecular docking studies of 1-phenyl-4-glycosyl-dihydropyridines as potent antileishmanial agents. *European Journal of Medicinal Chemistry.* 2010; 45(6):2381-2388.
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10. U.S. Singh, R. Shankar, A. Kumar, R. Trivedi, N. Chattopadhyay, N. Shakya, **S. Palne**, S. Gupta and K. Hajela. Synthesis and biological evaluation of indolyl bisphosphonates as anti-bone resorptive and anti-leishmanial agents. *Bioorganic Medicinal Chem*istry. 2008; 16(18): 8482-8491.
11. L. Gupta, A. Talwar, Nishi, **S. Palne,** S. Gupta, and P.M.S. Chauhan. Synthesis of marine alkaloid: 8,9-dihydrocoscinamide B and its analogues as Novel class of antileishmanial agents. *Bioorganic Medicinal Chem*istry. 2007; 17:4075-4079.
12. P.K. Murthy, S. Dixit, R.L. Gaur, R. Kumar, M.K. Sahoo, N. Shakya, S.K. Joseph, **S. Palne** and S. Gupta. Influence of Brugia malayi life stages and BmAFII fraction on experimental Leishmania donovani infection in hamsters. *Acta Tropica*. 2007; 106:81-89.

➢ Reviews

1. Gupta, S. and **Sane, Shraddha A.** Approaches towards Drug Development for Leishmniasis: A Review. *Drugs and Pharmaceuticals: Current R & D Highlights*. Leishmaniasis: CDRI, 2008; 31(4): 22-34.

List of Papers Presented in Symposia/Seminars

3rd International symposium on Current Trends in Drug Discovery Research, CTDDR, CDRI (Feb. 17-21, 2007)

1. Gupta S., Dixit S., Gaur R. L., Nishi, **Palne S.** and Murthy P. K. Hamsters co-infected with *Leishmania donovani* and *Brugia malayi*: Host responses.

International Conference on Advances in Drug Discovery Research (11thISCBC 2007) Dept. of Biochemistry, Dr. Babasaheb Ambedkar Marathwada Univ. Aurangabad, (Feb. 24-26, 2007)

2. **Palne S.,** Nishi, Shweta, Gupta S. and Narender T. *In Vitro* antleishmanial activity of few naturally occurring and synthetic chalcones.

19th National Congress of Parasitology–2007, Department of Zoology, Andhra University, Visakhapatnam (October 26-28, 2007)

3. Gupta S., Nishi, **Palne S.**, and Puri S. K. Antileishmanial efficacy of an 8-aminoquinoline drug - Elubaquine.
4. **Palne S.,** Srinivas, N., Nishi, Goyal N., Bhandari**,** K. and Gupta S. Antileishmanial activity of novel substituted azoles.

18th All India Congress of Zoology, University of Lucknow, Lucknow (Dec. 7-9, 2007)

5. Gupta S., Chandra N., Ramesh, **Palne S.**, Goyal N., Suryawanshi S. N. Terpenyl pyrimidines as antileishmanial agents.

20th National Congress of Parasitology, Department of Zoology, North- Eastern Hill University Shillong, India (November 3-5, 2008)

6. Nishi, Nagarapu S., **Palne S.**, Bhandari K., Gupta S. Antileishmanial activity of novel bis and mono imidazoles of cyclohexane.

World Leish 4–4th World Congress on Leishmaniasis, 2009, CDRI, Lucknow (Feb. 3rd-7th 2009)

7. **Sane S. A.,** Shakya N. and Gupta S. Antileishmanial efficacy of paromomycin and miltefosine combined with the immunomodulator picroliv.
8. Gupta S., Haq W., Shakya N. and **Sane S. A**. Immuno-chemotherapy of experimental visceral leishmaniasis using CpG-ODN in combination with Miltefosine: A preliminary study.
9. Nishi, **Sane S. A.** and Gupta S. Therapeutic Switching of primaquine in treatment of Visceral Leishmaniasis in combination with oral drug miltefosine and an immunomodulator.
10. Bhandari Kalpana, Srinivas Nagarapu, Marappu Vijay K., **Palne S.**, Nishi and Gupta Suman. The Antileishmanial Activity of Novel Tetrahydronaphtyl Azoles.

21st National Congress of Parasitology, Department of Zoology, Punjab University, Chandigarh-160014 (November 14-16, 2009)

11. Srivastava S., Marrapu V. K., Nagarapu, S., Verma A., **Sane S.A.,** Bhandari Kalpana and Gupta Suman. Antileishmanial activity of benzocycloalkyl azole oximino ethers.

14th ISCB International Conference (ISCBC-2010) on Chemical Biology for Discovery: Perspectives and Challenges, CDRI, Lucknow (Jan. 15-18, 2010)

12. **Sane S. A.,** Nishi, Haq W., and Gupta S. Augmentation of leishmanial chemotherapy in animal model using CpG-ODN in combination with Miltefosine. Abstract No. P-255, Page No. 176.

4th International Symposium on Current Trends in Drug Discovery Research (CTDDR-2010), CDRI, Lucknow, (Feb. 17-21, 2010)

13. Shakya N., **Sane S. A.,** Vishwakarma P. and Gupta S. Antileishmanial efficacy of Fluconazole and Miltefosine in combination with an immunomodulator-Picroliv. Abstract No. 86, Page No. 178, Abstract Published in Med Chem Res (2010) 19:S96.

➢ Patents

1. Novel substituted benzocycloalkyl azole derivatives as Antileishmanial agents- K. Bhandari, N. Sriniwas, **Shraddha Palne**, Nishi, and S. Gupta. Application No.0610Del 2008. NF No.0022 Nf/2008/IN.