Review of Literature

“All great achievements require time”

- Maya Angelou
CHAPTER 2

REVIEW OF LITERATURE

2.1 Chemotherapy

Leishmaniasis is a protozoan vector borne disease prevalent throughout the world and present in at least 88 countries. The parasite is transmitted by infected phlebotomine sandfly bites. While conventional therapies i.e. pentavalent antimonials, amphotericin B and pentamidine continue to play a major role, it is evident that new drugs or strategies must circumvent the limitations, such as a long-term parenteral administration, toxicity, the high cost in endemic countries and the emergence of resistance, that prevail. One of the most promising drugs is miltefosine, a new oral, approved alkylphospholipid for visceral leishmaniasis with only slight adverse effects. Although we have now this recent and encouraging advance, there is still a need to develop safe, efficient and affordable new treatments for the different clinical forms that exist. This review summarises conventional therapy and the current efforts in the discovery of drugs to treat leishmaniasis with the emphasis on drug combinations to enhance efficiency and prevent the emergence of resistance.

2.1.1 Agents Effective by Parenteral Route

2.1.1.1 Pentavalent Antimonials

N-methylglucamine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) have been used as a first line of treatment for VL since the 1940s. 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 1275mg 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).

![Chemical structure of Glucantime (A) and Pentostam (B)](image)

**Fig.2.1 Chemical structure of Glucantime (A) and Pentostam (B)**

Source: [www.aventis.com](http://www.aventis.com) and [www.sigmaaldrich.com](http://www.sigmaaldrich.com)

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) (Fig.2.1).

**2.1.1.2 Pentamidine Isothionate**

Pentamidine, an aromatic diamidine 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\(^+\) 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.

![Chemical structure of Pentamidine isothionate](www.scielo.br)

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) (Fig.2.2).

### 2.1.1.3 Amphotericin-B and its Formulations

Amphotericin B is a fungal antibiotic. Conventional Amphotericin B (fungizone®) is a macrolide polyene (Fig.2.3), characterised by hydrophilic polyhydroxyl and hydrophobic polyene aspects.

![Chemical structure of Amphotericin-B](www.ambisome.com)

It makes complexes with 24-substituted sterols, such as ergosterol in cell membrane, thus causing pores which alter ion balance and results in cell death (Roberts et al., 2003).
Leishmania parasites have same sterol (ergosterol) in their cell wall. It is a highly effective and highly costly treatment option for VL which is used as a first-line drug in India, where resistance to pentavalent antimonials is common. The best amphotericin B regimen is 15 doses of 1 mg/kg on alternate days (Pape, 2008). Oral amphotericin B is currently being assessed in Phase I as well as undergoing (animal) efficacy studies for VL. The drug could be considered for Phase II development by the end of 2009 (den Boer et al., 2009). Although this antibiotic has been widely used in the treatment of VL, there have been two small inconclusive studies on the emergence of amphotericin B resistance in L. infantum/HIV-infected cases in France. One study failed to find a change in sensitivity in promastigotes derived from isolates taken before and after the treatment of one patient (Durand et al., 1998). In contrast, a decrease in sensitivity was observed in isolates taken over several relapses from another patient (Di Giorgio et al., 1999). In a study, a micellar formulation of amphotericin B (AmB) solubilized with poloxamer 188 was evaluated against an AmB L. donovani-resistant line. Amastigotes showed 100 times less ED$_{50}$ against this formulation than that of the control AmB formulation (Espuelas et al., 2000). With the increasing use of amphotericin B in lipid formulations that have longer half-lives, the possibility of resistance cannot be ignored. AmBisome® accumulates in tissues and is only slowly released and excreted (Bekersky et al., 2000). This would theoretically increase the risk of resistant strains, but despite extensive use in VL, no in vivo resistance has yet been identified. This is likely to be due to the fact that parasites are killed very quickly by AmBisome®, and thus get little opportunity to develop into resistant strains. As well as-VL patients treated with amphotericin-B, care in a relatively well-equipped hospital for 30 days is required because of the risk of potentially serious side effects (especially renal toxicity). This makes it unfeasible for the treatment of patients. The main focus of the re-formulation of this highly active molecule is to increase solubility and thermal stability and decrease systemic toxicity of amphotericin B. A reduced cost of new amphotericin B formulations is also desired.

(i) Liposomal Amphotericin B

The efficacy of the antibiotic amphotericin B has been improved by the development of less toxic lipid formulations (L-ampB). Although originally developed for the treatment of
systemic mycoses, L-ampBs have been successfully exploited for VL with a high therapeutic index, short treatment courses and absence of side effects. It has an additional advantage of targeting the drug to infected macrophages of the liver and spleen. The liposomal amphotericin B formulation, AmBisome®, is registered treatment for visceral leishmaniasis (Meyerhoff, 1999). It has superior cure rates, lower relapse rates, few side effects and much better compliance and convenience and health care providers but its use in VL endemic regions is limited by cost. However, now it is available in India at a WHO negotiated price of 10% of its original price and therefore has become affordable within the national elimination program. This is very significant because now AmBisome® can be considered as a first line drug for VL (Sundar, S. unpublished work) A regimen of 20 mg/kg (total dose) of AmBisome® was recommended based on previous experience in different parts of the world in treating patients. However, lower doses may be sufficient for the Indian subcontinent. In India, in a small study, a single dose of 5 mg/kg of AmBisome® was effective in 91% of patients (den Boer et al., 2009). 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). Other commercial L-ampB formulations have been used for the treatment of VL.

(ii) Other Commercial Amphotericin B

Several other lipid forms have been evaluated in VL, such as Abelcet® (an amphotericin B lipid complex at the dose of 1-5 mg/kg per day) and Amphocil® (amphotericin B colloidal dispersion at the dose of 1 mg/kg of body weight). But none of these lipid formulations have yet been compared to AmBisome® in a clinical trial. Berman et al (1998) also reported effectiveness of unilamellar liposome formulation of AmBisome®, against VL in immunocompetent adults and children in Europe, Sudan, Kenya and India. Solid nanoparticles of amphotericin B deoxycholate have shown activity after intraperitoneal injection into L. donovani infected hamsters with 99% suppression of parasite replication in the spleen at a dose of 5mg/kg/day given for 5 days (Manadhar et al., 2008). A novel lipid based amphotericin B formulation has recently been reported as active after oral administration in L. donovani infected mice. Parasitic burden in the liver
was inhibited by 99.5% and 99.8% at doses of 10 and 20 mg/kg twice daily for 5 days (Wasan et al., 2009). N-(2-hydroxypropyl)-methacrylamide- GFLG-amphotericin B copolymer conjugates inhibited parasitic burden by up to 94% in the liver of *L. donovani* infected BALB/c mice after intravenous administration of 1mg/kg amphotericin B equivalent on 3 alternate days and by up to 99.6% at a dose of 3mg/kg amphotericin B equivalent (Nicoletti et al., 2009). This approach was extended to investigate poly (HPMA) - GFLG-amphotericin B-alendronic acid conjugates as potential combination therapeutics in models of VL (Nicoletti et al., 2010).

### 2.1.1.4 Paromomycin

PM or aminosidine is an aminoglycoside that was originally licensed by Farmitalia Carlo Erba as a broad spectrum parenteral antibiotic against bacteria and by Parke-Davis as an oral agent against intestinal protozoa. It was first used successfully in human VL in Kenya in the 1980s (Chunge et al., 1990) (Fig.2.4).

**Fig.2.4 Chemical structure of Paromomycin sulphate**

Source: [www.sigmaaldrich.com](http://www.sigmaaldrich.com)
WHO sponsored its development in India (Thakur et al., 1992) but later ran out of funding (Davidson et al., 2009). After that International Dispensary Association (The Netherlands) brought it back into production, it was adopted by iOWH and a large Phase III trial showed that a regimen of 21 days of 15 mg/kg given as daily intramuscular injections was highly effective with an excellent safety profile in India (Sundar et al., 2007). No nephrotoxicity, < 1% reversible ototoxicity and < 5% minor hepatotoxicities were reported. PM was licensed for VL in India in 2006, with registration in other VL endemic Asian and African countries planned. A Phase IV study in India is currently undertaken by iOWH, and PM is being evaluated in mono-and combination therapy in East Africa by DNDi. Preliminary results indicate that monotherapy in Sudanese VL has unacceptably low efficacy, whereas in Ethiopian VL, results were much better. PM should, therefore, only be used in combination with other drugs in Sudanese VL. PM resistance is readily induced in vitro (el-On J et al., 1991; Fong et al., 1994; Maarouf et al., 1998). Secondary resistance has also been observed after 60 days of PM injections in cutaneous leishmaniasis (Teklemariam et al., 1994). PM’s efficacy and safety in coinfectected patients are still unknown.

2.1.2 Agents effective by Oral Route

2.1.2.1 Miltefosine

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 analog of phosphatidylcholine (PC) - has a simple molecular structure (Fig.2.5). Croft and coworkers 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). Croft and coworkers 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 India in 2002 and Germany in 2004 as well as for cutaneous and visceral leishmaniasis in Colombia in 2004.
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 µM. 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).

**Pharmacological Class**

**Chemical Name:** 2-(hexadecoxy-oxido-phosphoryl) oxyethyl-trimethyl-azanium

**Empirical Formula:** C_{21}H_{46}NO_{4}P

**Molecular Weight** of miltefosine is 407.568 g/Mol.
Mechanism of Action

Although analogs of lipophospholipids (LPAs) were developed as anticancer agents, these compounds also have strong antiparasitic activity in vitro (Singh & 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 investigated 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 and 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 & 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 (WR6026) is an 8-aminoquinoline currently in clinical development by Glaxo Smith Kline for oral treatment of VL (Yeates, 2002) (Fig.2.6). Discovery of sitamaquine as antileishmanial agent was based on extensive efforts in synthetic chemistry at the Walter Reed Army Institute for Research (WRAIR) (Tekwani & Walker, 2006).

![Chemical structure of Sitamaquine](www.parasite.trends.com)

**Fig.2.6 Chemical structure of Sitamaquine**

Source: [www.parasite.trends.com](http://www.parasite.trends.com)

Recently results were reported from phase II dose ranging studies in India and Kenya. The overall cure rate at day 180 in the intention-to-treat-population was 83% in Kenyan patients (Wasunna et al., 2005) and 87% in Indian patients. Abdominal pain and headache were reported in the Kenyan study and vomiting, dyspepsia and cyanosis by the Indian investigators. Methemoglobinemia is associated with 8-aminoquinolines, but was only reported in Indian patients (Jha et al., 2005). Studies using rat and hamster liver microsomes have identified two major metabolites of sitamaquine, the desethyl and 4-CH$_2$OH derivatives, with evidence of cytochrome P-450 mediation (Theoharides et al., 1985; Yeates, 2002). Side chain oxidation and 5-hydroxylation have been identified as important steps in the metabolic pathway of 8-aminoquinolines (Idowu et al., 1995; Yeates, 2002). Presystemic elimination of sitamaquine in the liver with low systemic availability was observed in Beagle dogs (Taylor et al., 1991). The elimination half-life of
sitamaquine in humans is reported as 26.1 hours. The major urinary metabolite in humans is the 4-CH$_2$OH derivative with a reported elimination half-life of 29.1 hours. A minor metabolite in humans is the desethyl species (Yeates, 2002). Metabolites may be linked to efficacy and toxicity of this compound. Sitamaquine induced morphological changes in intracellular *L. tropica* amastigotes and host macrophages (Langreth *et al*., 1983). Collapse of mitochondrial membrane potential in *L. donovani* promastigotes has also been shown (Vercesi *et al*., 1992) as well as alkalisation of acidocalcisomes (Vercesi *et al*., 2000). Recently anti-leishmanial activity has been demonstrated as unrelated to sitamaquine accumulation in this organelle (Lopez-Martin *et al*., 2008). The interaction of sitamaquine with membrane lipids of *L. donovani* promastigotes has been assessed and described as a two-step process (Duenas-Romero *et al*., 2007).

### 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. Their efficacy against *L. tropica* was first reported by Berman (1981). Leishmania cell membrane synthesis involves the conversion of squalene to ergosterol. Azole exerts its effect by selectively inhibiting the cytochrome P450 enzyme 14 α-demethylase. The result is a loss of normal sterols and the accumulation of 14 α-methyl sterols within the cell (Mahmoud *et al*., 1999) (Fig.2.7).

![Fig.2.7 Mechanism of action of azoles](www.HIVwebstudy.org)
(i) Fluconazole

It was first synthesised and patented in 1982 as an antifungal agent and used in the treatment and prevention of superficial and systemic fungal infections (Fig.2.8). In a bulk powder form, it appears as a white crystalline powder, and it is very slightly soluble in water and soluble in alcohol. Fluconazole has also rarely been associated with severe or lethal hepatotoxicity and liver function tests are usually performed regularly during prolonged fluconazole therapy. In addition, it is used with caution in patients with pre-existing liver disease (http://en.wikipedia.org/wiki/Fluconazole).

![Fig.2.8 Chemical structure of Fluconazole](http://en.wikipedia.org)

**Pharmacological Class**

**Chemical Name:** 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol

**Empirical Formula:** C\textsubscript{13}H\textsubscript{12}F\textsubscript{2}N\textsubscript{6}O

**Molecular Weight** of fluconazole is 306.271 g/Mol.

**Pharmacokinetics**

Following oral dosing, fluconazole is almost completely absorbed within two hours. Bioavailability is not significantly affected by the absence of stomach acid. Concentrations measured in the urine, tears and skin are approximately 10 times the plasma concentration,
while saliva, sputum and vaginal fluid concentrations are approximately equal to the plasma concentration, following a standard dose range of between 100 mg and 400 mg per day. The elimination half-life of fluconazole follows zero order kinetics and only 10% of elimination is due to metabolism, the remainder is excreted in urine and sweat.

**Dosage used in Fungal Infection**

Dosage, varies with indication and between patient groups, ranging from: a two week course of 150 mg/day for vulvo-vaginal candidiasis, to 150–300 mg once weekly for resistant skin infections or some prophylactic indications. 500–600 mg/day may be used for systemic or severe infections, and in urgent infections such as meningitis caused by yeast, 800 mg/day dose has been used. Paediatric doses are measured at 6-12 mg/kg/day.

**Side Effects**

Adverse drug reactions associated with fluconazole therapy include (Rossi, 2006):

*Common (≥1% of patients):* rash, headache, dizziness, nausea, vomiting, abdominal pain, diarrhoea, and/or elevated liver enzymes.

*Infrequent (0.1–1% of patients):* anorexia, fatigue, constipation

*Rare (<0.1% of patients):* oliguria, hypokalaemia, paraesthesia, seizures, alopecia, Stevens-Johnson syndrome, thrombocytopenia, other blood dyscrasias, serious hepatotoxicity including hepatic failure, anaphylactic/anaphylactoid reactions.

*Very rare:* prolonged QT interval, torsades de pointes.

**Precautions**

Fluconazole has also rarely been associated with severe or lethal hepatotoxicity and liver function tests are usually performed regularly during prolonged fluconazole therapy. In addition, it is used with caution in patients with pre-existing liver disease. High concentrations of fluconazole have been detected in human breast milk from patients
receiving fluconazole therapy, thus its use is not recommended in breast feeding mothers. Fluconazole therapy has been associated with QT interval prolongation, which may lead to serious cardiac arrhythmias.

(ii) Ketoconazole

Ketoconazole was discovered in 1976 and released in 1981. It is usually prescribed for topical infections such as athlete's foot, ringworm, candidiasis (yeast infection or thrush), and jock itch (Fig.2.9). The over-the-counter shampoo version can also be used as a body wash for the treatment of Tinea versicolor (http://en.wikipedia.org/wiki/Ketoconazole).

![Chemical structure of Ketoconazole](http://en.wikipedia.org/wiki/Ketoconazole)

**Fig.2.9 Chemical structure of Ketoconazole**

Source: en.wikipedia.org

**Pharmacological Class**

**Chemical Name:** 1-[(4-(4-[(2R,4S)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy)phenyl)piperazin-1-yl]ethan-1-one

**Empirical Formula:** C₇₆H₂₈Cl₂N₄O₄

**Molecular Weight** of ketoconazole is 531.431 g/mol.

**Pharmacokinetics**

Ketoconazole is very lipophilic, which leads to accumulation in fatty tissues. Ketoconazole is best absorbed at highly acidic levels, so antacids or other causes of decreased stomach
acid levels will lower the adsorption of drug when taken orally. Absorption can be increased by taking it with an acidic beverage, such as Cola (Chin et al., 1995).

**Dosage used in Fungal Infection**

In adults, recommended starting dose of ketoconazole is administration of 200 mg. In very serious infections or if clinical responsiveness is insufficient within the expected time, the dose of ketoconazole may be increased to 400 mg once daily. However, children over 2 years of age, a single daily dose of 3.3 to 6.6 mg/kg have been used. Ketoconazole has not been studied in children under 2 years of age.

**Side Effects**

The side-effects of ketoconazole are sometimes used to treat non-fungal problems. The decrease in testosterone caused by the drug makes it useful for treating prostate cancer and for preventing post-operative erections (Evans et al., 2004) following penile surgery. Another use is the suppression of glucocorticoid synthesis, where it is used in the treatment of Cushing's disease (Paola et al., 1986). These side effects have also been studied for use in reducing depressive symptoms (Wolkowitz et al., 1999) and drug addiction (Goeders et al., 1998) however; it has not succeeded in either of these roles (Ward et al., 1998; Malison et al., 1999).

**Precautions**

Ketoconazole should be used only if clearly needed during pregnancy. It should be used with caution in patients with any pre-existing illnesses or any allergy. Ketoconazole should be used with caution in the children under 2 years of age. It should also be used with caution in the patient with Azole hypersensitivity.

**Application of Azoles in the Field of Leishmaniasis**

The results from in vitro studies that have investigated the intrinsic differences in sensitivity of *Leishmania* species to sterol biosynthesis inhibitors have produced
contradictory data. In a comparative study on the sensitivity of promastigotes to ketoconazole, *L. donovani*, *L. braziliensis* and *L. amazonensis* were found to be more sensitive than *L. aethiopica*, *L. major*, *L. tropica*, and *L. mexicana* (Beach et al., 1998). However, in contrast, Rangel et al (1996) observed that *L. braziliensis* was relatively insensitive to ketoconazole and the bistriazole D087, whereas *L. mexicana* was sensitive to ketoconazole. Both sets of results differ from those of an earlier study using an amastigote-macrophage model, which showed that *L. donovani* was more sensitive to ketoconazole than *L. mexicana* or *L. major* (Berman, 1981). The lack of concordance is probably due to different assay conditions, already shown to greatly influence antifungal activities, as well as the ability of amastigotes to salvage sterols, such as cholesterol, from host cell macrophages. This factor can reduce the sensitivity of this life cycle stage to azoles (Roberts et al., 2003). A number of clinical studies have suggested that these sterol biosynthesis inhibitors are more effective against *L. major* and *L. mexicana* infections than against *L. donovani* or *L. braziliensis* infections. One placebo-controlled trial on the treatment of CL showed that *L. mexicana infections* (89%) were more responsive than *L. braziliensis infections* (30%) to ketoconazole (Croft et al., 2006). 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). Fluconazole was used clinically due to its good oral absorption, minimal protein binding character, few adverse reactions and similar mode of action to that of Amphotericin B (Jha 1998). Sundar et al (1996) reported that fluconazole elicited clinical and parasitological cure in only 50% of cases when it was used at the dose of 6 mg/kg/day for 30 days, but these had relapses within 2 months. Fluconazole was used clinically in combination with allopurinol in the treatment of VL (Torres et al., 1996) and this combination is used successfully by Colakoglu et al (2006) in the patients of VL with renal failure. Against CL it has also shown promise (Mussi & Fernandes 2007). In a study in Saudi Arabia, fluconazole showed a cure rate of 79% in patients of CL caused by *L. major* (Alrajhi et al., 2002). Combination of ketoconazole and allopurinol was used clinically by Hueso et al., (1999) and Llorente et al (2000) in the patients of VL treated with glucantime who developed abdominal pain, nausea, vomiting
and acute pancreatitis respectively. Halim et al. (1993) also reported that a case of a renal transplant recipient who developed pancreatitis during stibogluconate treatment for VL was also successfully treated with this combination.

**Clinical Resistance**

A number of clinical studies have suggested that these sterol biosynthesis inhibitors are more effective against *L. major* and *L. mexicana* infections than against *L. donovani* or *L. braziliensis* infections. One placebo-controlled trial on the treatment of CL showed that *L. mexicana* infections (89%) were more responsive than *L. braziliensis* infections (30%) to ketoconazole (Navin et al., 1992).

**Mechanisms of Resistance**

Extensive studies in *Candida* spp. have shown that mutations at both the active site and heme cofactor site of cytochrome P450 sterol 14-demethylase (CYP51) can result in reduced sensitivity to azoles. Clinical resistance in *C. albicans* isolates has been shown to be due to drug efflux following up regulation of ABC and multidrug transporters as well as up regulation of several ERG genes that code for enzymes in the sterol biosynthesis pathway. There have been no published experimental studies on acquired resistance in *Leishmania* spp., but resistance to fluconazole was shown to be rapidly induced *in vitro* in the related parasite *Trypanosoma cruzi* (Buckner et al., 1998).

**2.1.2.4 Buparvaquone**

Buparvaquone is a hydroxynaphthoquinone, which is currently marketed as Butalex® for the treatment of theileriosis in cattle (Fig.2.10). For the first time Croft et al. (1992) has been tested BPQ against *L. donovani* infected BALB/c and observed a 62% suppression of hepatic amastigotes burden. Its potent *in vitro* activity was confirmed against a range of *Leishmania* spp. with EC_{50} values for the intracellular amastigote stage in the low micromolar to nanomolar range. The same study investigated water soluble phosphate prodrugs of buparvaquone and reported potent *in vitro* activity against CL and VL causing *Leishmania* species (Mantyla et al., 2004a).
Buparvaquone oxime derivatives were also investigated, but displayed lower *in vitro* activity against *L. donovani* than the parent compound (Mantyla *et al.*, 2004b). The prodrug approach is an effective way of improving oral bioavailability of poorly soluble drugs by chemical derivatization to more water soluble compounds. It is also used to improve topical drug delivery. Formulations for topical delivery of buparvaquone and a prodrug (3-phosphono-oxymethyl-buparvaquone) have been developed and characterized in *in vitro* human and mouse skin models (Garnier *et al.*, 2007a). Efficacy of topical formulations and phosphate prodrugs of buparvaquone in *in vivo* models of VL and CL has been reported (Garnier *et al.*, 2007b).

### 2.2 Drug Screening in VL

#### 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 a semi-*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
The requirement of test compound is very minute
The turnover of screening results are quick and
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) 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 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 $1-2 \times 10^6$ per ml of cultivation medium and the drugs in appropriate concentrations are added to the experimental culture. The inhibition of promastigote multiplication is assessed after approximately 3 days, during which the control organism multiply 3-6 times. The technique is simple and easily applicable. However, the metabolism and ecology of promastigote differ so widely from those of amastigote (target form) that screening data obtained from 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) 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 (Fig.2.11). Axenic amastigotes are obtained by subjecting promastigotes to pH change at 37°C. A direct comparison of the drug susceptibility towards standard antileishmial 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 & 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 & 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.

![Axenic amastigotes](www.jbc.org/content)

**Fig.2.11 Axenic amastigotes (100X under oil immersion lens)**
Source: [www.jbc.org/content](http://www.jbc.org/content)

(iii) **Intracellular 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.

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 microscopical counting of percentage of infected cells or number of amastigotes per macrophage (Neal &Croft, 1984) or colorimetric or fluorimetric 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 (as described under) must ensure that the confounding effects of drug activity on both parasite and host cell number are considered (Croft *et al*., 2006a).

(a) In Tumour Macrophages
Mattock & 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).

(b) Mouse Peritoneal Macrophages
For *in vitro* drug testing, Peters *et al* (1980) employed infected mouse peritoneal macrophages, which were also used by Neal & Mathew (1982), Neal & 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.

(c) 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 & Koromyslov, 1982). Otherwise, theoretically this model is most appropriate because it resembles best with the clinically infected macrophages.
2.2.1.2 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.

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 $2 \times 10^7$ *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 & Kirkley, 1972). The percent inhibition was calculated for all drug-treated groups in relation to untreated group, and ED\textsubscript{50} are calculated.

**Hamster Model**

Although many hamster species are susceptible to *L. donovani* infection (Smyly & Young, 1924), the Syrian golden hamster (*Mesocricetus auratus*) establishes a good model for VL and provides a more synchronous infection in the liver and spleen that can develop into a chronic non-cure infection more similar to human VL (Farrell, 1976; Gifawesen & Farrell, 1989; Hommel et al., 1995) (Fig.2.12).

Hamsters are infected intracardially. 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 & 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 & 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 & 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.

**Fig.2.12 Syrian golden hamster**
Source: www.trinifieds.com

**Rat Model**
The cotton rat (*Sigmodon hispidus*) represents one of the most susceptible animal hosts for *L. donovani* (Fulton & 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 & Mansour (1973) and McKinney & 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.

(ii) **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 (Riouxi *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).

(iii) 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 & 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 & Wyler, 1980; Berman, 1984; Berman & 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 & 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 & 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 color that can be measured at 570 nm in the visible range or in the fluorometric UV range at 590 nm. The oxidized blue state can only be read at 600 to 630 nm in the visible range, and it is not fluorometric. The transformation of 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 & 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 & 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
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 & Dube, 2004; Mehta et al., 2008) wherein the fluorescence intensity in parasites decreased with time in the absence of geneticin sulphate (antibiotic G418), 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 & 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 & Wilson, 2005).

### 2.2.2.7 β-Lactamase

Buckner & 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, thereby 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™ 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 & 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 & Subramani, 1988) represents one of the most efficient biological reporter molecules, which allow monitoring host-microbe interactions (Valdivia & 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 CSIR-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 or (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 & 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 acticity of the test compound to that of glucantime) for each test compound was calculated by the following formula.

\[
\text{Glucantime index (G.I.)} = \frac{SD_{90} \text{ for Glucantime}}{SD_{90} \text{ for test compound}}
\]

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

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 Beveridge 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 (p.t.) in addition to on day 7 and day 14 p.t. thus, facilitating assessment of the status of parasite at different intervals. A critical appraisal of the screening techniques by Gupta et al (1992) also shows that none of them is able to provide comprehensive information about the total efficacy of a potential drug. This is because the total effect of a drug is depending on two factors, (a) the effect of drug on the parasites, and (b) on host immune system. In Stauber’s and Hanson’s techniques, the assessment is based on the effect of a potential drug on day 1 - 3 p.i. and that too on the parasite in liver only. This is far from actual situation in clinical practice where, the VL cases are more chronic and the parasites are located in deeper organs like spleen and bone marrow. Further, these methods completely ignore the host immune system. Mikhail and Mansour (1975) started treating animals on day 15 p.i., the treated and control animals were sacrificed on day 60 post-infection. This technique can detect delayed action of a drug quite well. Beveridge’s (1963) method is more logical as the pre-treatment parasitic burden is assessed by spleen biopsy to select experimental animals carrying similar parasitic load. However, the animals are sacrificed on day 7 post-treatment (p.t.). It is, therefore, impossible to assess the delayed action of drugs. Guru et al (1989) modified the technique where the delayed action of drugs can also be assessed conducting repeated spleen biopsies on the same animal at different intervals of day 7, 14, and 28. This is more rational as it 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 blabbing, 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. Bcl-2 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") (Fig.2.13).

![Fig.2.13 Components of the intrinsic or mitochondrial pathway](source: www.users.rcn.com)

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 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.

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.2.14).

Fig.2.14 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 anti-leishmanial 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

Apoptotic cells are characterized by morphological markers such as cell shrinkage, phosphatidylserine (PS) exposure, mitochondrial membrane depolarization, DNA fragmentation, membrane blabbing and packaging of cell contents into apoptotic bodies. In the present study following two markers have been used.

(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 ΔΨ) 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′-tetraethylbenzimidazolylcarbocyanine 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 effector 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, Ouaissi, 2007).

2.4.1 Cell Mediated Immune Response

Leishmania is an intracellular parasite so CMI constitutes an important role in host defense 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. 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 (Fig.2.15). 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 effector 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.2.15 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. 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.2.16).

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-labelled 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 4½ 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

(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 (Fig.2.17).

![Fig.2.17 Generation of reactive oxygen and nitrogen Species](source: www.chinaphar.com)

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).
(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; Wulf, 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$_2$O$_2$ (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-\(\gamma\) (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 and PTX, L-NAME, NaN3 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 multi-layered 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 & 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, opsonisation by complement improves parasite survival (Mosser, et al., 1987; Mosser & Edelson, 1987). The CR4, fibronectin receptor, mannose receptor and the advanced glycosylation end-product receptor have also been implicated in invasion (Alexander & 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.

### 2.5 Combination Therapy for Leishmaniasis

#### 2.5.1 Introduction

Combining drugs is an established approach for treating several infectious diseases, and this practice is gradually permeating the world of tropical parasitic diseases. In visceral leishmaniasis drug combinations have been attracting attention mainly because of resistance to first-line antimonials, the need to prolong drugs, useful therapeutic lifespan, practicalities (shortening length of treatment regimens, better adherence) and improved cost-effectiveness. New drugs with novel mechanisms of action will only come from discovery research programs, and there is at present no new antileishmanial drug in clinical development, which means a gap of possibly 10 years to be filled before another potential antileishmanial drug could be brought to patients. Against this scenario, there are options today for combination treatments for visceral leishmaniasis (Bryceson, 2001; Alvar et al., 2006; Croft et al., 2006; den Boer & Davidson, 2006; Singh et al., 2006b; Sundar & Olliaro, 2007, Olliaro, 2010). 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 & 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 chance 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). Bryceson advocated the examination of combinations of strong antileishmanials with week drugs (for example, azoles). Despite of remarkable work done on combination therapy for Leishmaniasis (Chunge et al., 1990; Murray & Hariprashad, 1996), it has not yet been adapted as standard treatment. Limitation was unavailability of effective antileishmanial drug. But with paromomycin and miltefosine, this picture changed. A combination therapy also needs to be evaluated for safety and optimized for either concomitant or sequential administration of component drugs (Bryceson, 2001).

2.5.2 Pre-Clinical Data on Combination Therapy

Few preclinical data on the efficacy and safety of combination therapies for visceral leishmaniasis are available. 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 (an oral aminoquinoline) (Seifert & 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 & Croft, 2006).

2.5.3 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). Sequential treatments with liposomal amphotericin B single dose and miltefosine (liposomal amphotericin B 5 mg/kg followed by 7, 10 or 14 days of miltefosine 100 mg/kg/day or liposomal amphotericin B 3.75 mg/kg followed by 14 days of miltefosine) were all highly effective in a phase 2 dose-finding study in Bihar with adaptive (triangular) design (n=4181). The 9 months cure rates were 98%, 98%, 96% and 96%, respectively (liposomal amphotericin B 5 mg/kg single dose alone was 91% effective). It is the first trial to show the efficacy of short, sequential treatments with liposomal amphotericin B followed by miltefosine. It was also the first to use a triangular study design in leishmaniasis (Sundar et al., 2008). All combinations were highly efficacious (more than 95% of patients cured) and well tolerated, irrespective of the duration of miltefosine treatment. A phase 2 trial studying the combination of liposomal amphotericin B (5 mg/kg) with miltefosine for 14 days is underway in India and planned in Bangladesh (B Arana, WHO Special Programme for Research and Training in Tropical Diseases, personal communication, Nov 3, 2009) (Anon, 2010a). Several short
combinations are being studied in a large non-inferiority phase 3 trial in India, (Anon, 2010b) which has now moved into its second stage will be started in Bangladesh and Nepal; the first results from India are expected by 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., 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; Melaku et al., 2007; Wakabi, 2007; Davidson et al., 2009; Anon, 2010c). This phase 3 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, 2010c). 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 (Berman et al., 1998; Mueller et al., 2006, 2008; Ritmeijer et al., 2006) adding children into the study.

2.5.4 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. The first projections of the cost-effectiveness of antileishmania treatments in India (direct costs only) including combinations (sequential treatment of liposomal amphotericin B followed by miltefosine) (Olliaro et al., 2009). Another findings on the cost-effectiveness of combination therapies
in India, Nepal, and Bangladesh, showed combination therapies to be a viable alternative to monotherapies, with liposomal amphotericin B and paromomycin the best combination economically (Meheus et al., 2009, Meheus et al., 2010).

2.5.5 Impact on Drug Resistance

The problem of drug resistance in visceral leishmaniasis has been extensively reviewed elsewhere (Croft et al., 2006) Treatment failure can manifest as initial treatment failure (failure to clear parasites at the end of the treatment course) or relapse (reappearance of parasites after initial cure, usually within 6 months of follow-up). 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 hyperendemic 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 pretreatment 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).

2.6 Approaches used in the Treatment of VL

2.6.1 Immunomodulators used in Combination Therapy

Instead of relying on drugs to reduce the parasite burden of leishmaniasis, and waiting for the effector immune response to develop in time to control the parasites, immunotherapy in conjunction with chemotherapy can rapidly induce the effector immune response. A low-dose or short course of an effective drug in combination with an immunomodulator has been a successful approach for effective treatment of leishmaniasis and might be sufficient to induce a quick and lasting recovery. Drug toxicity and the emergence of resistance could also be dramatically reduced compared with present long-term monotherapy. Immunotherapy could be an effective addition to chemotherapy for leishmaniasis.

Fig.2.18 Schematic representation of chemotherapy plus immunotherapy in leishmaniasis

The natural history of leishmaniasis is depicted in Fig.2.18 in a simplified schematic model applicable to VL and some forms of CL, with the assumption that there is a direct relationship between parasite load and disease. In an immunocompromised host the disease
returns upon stopping treatment (Fig. 2.18. A). In cured or asymptotically infected individuals, fulminating disease appears after immunosuppressive drugs or HIV infection. However, strong immunity is developed following successful recovery in immunocompetent individuals (Fig. 2.18. B). Hence, the protective immune response is an important part of recovery from leishmaniasis. The available treatment options are far from satisfactory as they are either expensive (amphotericin B) or toxic (antimonials), or resistant parasites have either emerged or are imminent with monotherapy (miltefosine and paromomycin). One solution is to combine these drugs to allow shorter, less toxic and more affordable treatment. This approach is being addressed by WHO, the Drugs for Neglected Diseases initiative and their endemic country partners. Another alternative in combination therapy is immunochemotherapy, whereby a low-dose or short course of an effective drug is given with one injection of an immunomodulator to quickly induce the effector immune response (Fig. 2.18.C) (Musa et al., 2010).

The efficacy of treatment of Leishmania is compromised due to suppression of immune function during the course of infection (Bogdan, 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–Guérin), 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 imiquimod (Buates & 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). Smith et al (2000) evaluated antileishmanial efficacy of “tucaresol” against *L. donovani* in BALB/c mice and found it to be moderately effective. 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. 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 Sb⁵ were undertaken. With Sb⁵ it was reported to be useful in treating severe or Sb⁵ refractory VL in Brazil, however, in India in a large randomized study comparing Sb⁵ alone with Sb⁵ plus IFN-γ for 15 or 30 days had disappointing results as the final cure rate with Sb⁵ plus IFN-γ was 42 and 49 per cent, respectively (Sundar and Chatterjee, 2006). Based on these findings, we have explored the effect of an immunomodulator and hepatoprotective agent picroliv in combination with azoles and standard antileishmanial, miltefosine in hamster/*L. donovani* model. We have recently reported enhancement of therapeutic efficacy of miltefosine in combination with CpG-ODN as immunomodulator in rodent models of *L. donovani* (Sane *et al.*, 2010). Along with this we have also studied the effect of two another immunomodulators namely Pam3Cys and tuftsin in combination with miltefosine in mouse.

### 2.6.1.1 Immunomodulators used in Present Work

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).

**Ia) 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) (Fig.2.19. a, b). 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.

![Fig.2.19 Chemical Structure (a) Picroside-I (b) Kutkoside](image)


**Ib) 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).

Ic) 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).

Id) Pharmacological Actions
Most of the research work on P. kurroa has focused on its hepatoprotective, anticholestatic, antioxidant, antiinflammatory and immune-modulating activities.

Ie) 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 & 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).

If) 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).

Ig) 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 hepatotoxins. 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).
Ih) 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 Picrorhiza kurroa extracts have an inhibitory effect on proinflammatory cells such as neutrophils, macrophages and mast cells (Pandy & 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).

Ii) 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 & Kuttan 2000).

Ij) 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 waiting for phase III clearance (Negi et al., 2007) and will be very soon in the market for human use.

Ik) 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.
II) 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.

II) Tuftsin
Tuftsin is a physiologic tetrapeptide, TKPR which is an integral component of the leukophilic immunoglobulin G (residues 289 to 292) and is released physiologically as the free peptide fragment after enzymatic cleavage of the Fe-domain of the heavy chain. It is produced primarily in the spleen.

IIa) Discovery
Tuftsin, a naturally occurring peptide with the sequence- Thr$^{289}$-Lys$^{290}$-Pro$^{291}$-Arg$^{292}$, was originally described in 1970 by Najjar & Nishioka as a phagocytosis-stimulating peptide derived from the proteolytic degradation of IgG 2 (Siemion & Kluczyk, 1999). It was named after Tufts University where the peptide was discovered.

IIb) Structural Characteristics
Tuftsin is an immunostimulatory peptide with reported nervous system effects as well (Fig.2.20). Tuftsin and a higher affinity antagonist, TKPPR, bind selectively to neuropilin-1 and block vascular endothelial growth factor (VEGF) binding to that receptor. Dimeric and tetrameric forms of TKPPR had greatly increased affinity for neuropilin-1 based on competition binding experiments. On endothelial cells tetrameric TKPPR was found to inhibit the VEGF165-induced autophosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) even though it did not directly inhibit VEGF binding to VEGFR-2. Homology between exon 8 of VEGF and TKPPR suggests that the sequence coded for by exon 8 may stabilize VEGF binding to neuropilin-1 to facilitate signalling through VEGFR-2. Given the overlap between processes involving neuropilin-1 and tuftsin, we
propose that at least some of the previously reported effects of tufts in are mediated through neuropilin-1 2 (von Wronski et al., 2006).

IIc) Pharmacological Class

Chemical Name: N (2)-(1-(n (2)-l-threonyl)-l-lysyl)-l-prolyl)-l-arginine

Molecular Formula: C_{21}H_{40}N_{8}O_{6}

The Molecular Weight of tufts in is 500.593 g/Mol.

![Chemical structure of Tufts in](source: www.sigmaaldrich.com)

IIId) Tufts in Analogs

Cyclic analogs of the physiological immunostimulating peptide tufts in, ctuf-G {cyclo (Thr-Lys-Pro-Arg-Gly)} and ctuf-D {cyclo (Thr-Lys-Pro-Arg-Asp)} were synthesized based on molecular modeling studies, and assayed for the ability to stimulate phagocytosis by human polymorphonuclear leukocytes. As predicted, the synthesis of ctuf-D resulted in two isomers with the correct molecular mass and amino acid composition. In phagocytosis assays, tufts in, ctuf-G and two isomers of ctuf-D showed the usual bell-shaped activity profiles. The optimum concentration of ctuf-G was 50-fold less than that of tufts in, whereas the degree of stimulation was similar. One isomer of ctuf-D was almost inactive, and the other ctuf-D exhibited the same degree of phagocytosis as tufts in but its optimum concentration was 5-fold lower. The enhanced potency of ctuf-G and one isomer of ctuf-D may be due to conformational effects and/or to the possibility that these cyclic peptides are resistant to proteolytic degradation.
IIe) Related Peptides
Numerous proteins and peptides contain within them tuftsin or tuftsin-like sequences. Some of these, such as C-reactive protein, are known mediators of inflammation. In addition, two other pro-inflammatory peptides, substance P and neurotensin, both contain tuftsin-like sequences, stimulate phagocytosis, and can compete with [3H] tuftsin for binding to macrophages. Moreover fragments of substance P and neurotensin containing the tuftsin-like sequences maintain their tuftsin-like activity even though their interactions with the known substance P and neurotensin receptors are greatly reduced. Although a number of tuftsin-like peptides reportedly share the biological activity of tuftsin, several similar peptides, such as TKPPR, are potent tuftsin antagonists, indicating that there are highly specific structural requirements for tuftsin agonists (von Wronski et al., 2006).

IIf) Mode of Action
Cultured human aortic and umbilical vein endothelial cells possess tuftsin receptors and that in these cells the binding target for tuftsin is neuropilin-1. Since neuropilin-1 plays a critical role in the immune, vascular, and nervous systems and interacts with a number of different ligands, cell surface receptors, adhesion proteins, and intracellular proteins, it was proposed that at least some of the previously reported effects of tuftsin are mediated through neuropilin-1 (von Wronski et al., 2006).

IIg) Functions performed by tuftsin
Several reports indicate that tuftsin or tuftsin-like peptides exert multiple stimulatory effects on a subset of immunologic effector cells, including enhanced migration/chemotaxis, enhanced phagocyte respiratory burst, enhanced antigen presentation, and other undefined immunologic effects that result in increased antimicrobial and antitumor activities by immune cells. Additionally tuftsin is reported to have effects on the nervous system, including induction of analgesia and inhibition of axonal regeneration (von Wronski et al., 2006).

IIg) Pathology
Tuftsin deficiency, either hereditary or following splenectomy, results in increased susceptibility to certain infections e.g.: caused by capsulated organisms as: H. influenza, pneumococci, meningococci and salmonella. Tuftsin activity is reduced in cirrhosis. This
contributes to the defective phagocytic activity of neutrophil granulocytes and is related to the impairment of splenic function (Foschi et al., 2005). Tuftsin activity was significantly lower in patients with AIDS, AIDS-related complex and in those who had undergone splenectomy compared with healthy volunteers. Tuftsin deficiency may contribute to the risk of bacterial infection in symptomatic HIV-positive individuals (Corazza et al., 1991).

IIh) Need for the preparation of Palmitoyl Tuftsin

Tuftsin, due to its hydrophilic character, cannot be grafted on the surface of liposomes without being attached to a sufficiently long hydrophobic anchor. Structure–function studies of this tetrapeptide indicate that its binding and subsequent activation of the mononuclear phagocyte system (MPS) is dependent upon rather strict conservation of its molecular structure. Thus, modifications of the peptide at its N-terminus or within the chain lead to a significant reduction or even loss of its biological activity (Fridkin & Gottlieb, 1981). However, the activity is largely retained if modifications are restricted only to the C-terminus (Gottlieb et al., 1982).

All the modifications are, thus, limited to the carboxyl group of the Arg residue. Direct attachment of a fatty acyl group to the Arg residue, without any spacer arm, leads to modified tuftsin, which does not allow formation of liposomes, presumably due to perturbation of the phospholipid polar head group packing by the bulky Arg residue (Singhal et al., 1984). This problem is, however, circumvented by introducing an ethylenediamine spacer arm between the Arg residue and the hydrophobic anchor (Fig.2.21). Liposomes containing palmitoyl tuftsin specifically recognize macrophages and PMN leukocytes (Singhal et al., 1984). Treatment of macrophages with these liposomes considerably increases their respiratory burst activity (Singh et al., 1992). Pretreatment of animals with tuftsin-bearing liposomes enables the animals to resist malaria (Gupta et al., 1986), leishmania (Guru et al., 1989), and fungal (Owais et al., 1993) infections. In addition, delivery of antileishmanial (Agrawal et al., 2002; Guru et al., 1989), antitubercular (Agarwal et al., 1994), antifungal (Owais et al., 1993), and antifilarial (Owais et al., 2003) drugs in liposomes containing palmitoyl tuftsin (Molecular mass: 781 g/M) is shown to increase the therapeutic efficacy of drugs against these infections. We are using palmitoyl tuftsin in our study.
II) Application of Tuftsin in Leishmania Therapy

Palmitoyl Tuftsin is also used in the field of leishmania chemotherapy. Guru et al (1989) demonstrated that efficacy of sodium stibogluconate against *L. donovani* infections was markedly enhanced by encapsulating this drug in tuftsin-bearing liposomes thus offering an additional advantage over the use of tuftsin-free liposomes as drug carriers in leishmania therapy. The macrophage-activating tetrapeptide tuftsin was able to activate murine peritoneal macrophages to express nitric oxide (NO) synthase and to produce NO which is able to kill the amastigotes of the intracellular protozoan parasite *L. major* (Cillari et al., 1994). Superior chemotherapeutic efficacy of amphotericin B in p-tufts-in-bearing liposomes against *L. donovani* infection in hamsters was reported by Agrawal et al (2002). These results further demonstrate the usefulness of p-tufts-in-bearing liposomes as drug vehicles in treatment of the macrophage-based infections that have been reviewed later by Agrawal et al (2000).

III) Pam3Cys

The lipopeptide tripalmitoyl-S-glycerylcysteine (Pam3Cys) is derived from the N-terminal part of bacterial lipopeptides. Bacterial lipoproteins are a family of proinflammatory cell wall components found in both Gram positive and Gram negative bacteria. The stimulatory activity of bacterial lipoproteins resides in their acylated amino terminus. It is a macrophage and polyclonal B-lymphocyte activator (Bessler et al., 1985; Hoffmann et al., 1989). Derivatives of Pam3Cys constitute highly potent, nontoxic immunoadjuvants, and lipopeptide–antigen conjugates have found important applications as novel fully synthetic low-molecular-weight vaccines.
IIIa) History
The first work on synthetic lipopeptides was based on the immunologically active N-terminal moiety of the principal lipoprotein of *Escherichia coli*, also known as Braun’s lipoprotein (Braun, 1975). The tripalmitoyl-S-glycerylcysteinyll- (Pam3Cys) scaffold mediates attachment to the cell membrane, internalization into the cytoplasm, and activates macrophages to secrete cytokines (Hoffmann *et al.*, 1988; Wolf *et al.*, 1989; Metzger *et al.*, 1993). Synthesis of synthetic analogues (sLP) and the N-terminal part of the lipoprotein of *E. coli* were done for the first time in 1983 (Wiesmüller *et al.*, 1983). They act as potent immunoadjuvants *in vivo* and *in vitro*. Synthetic viral peptides covalently linked to Pam3Cys were demonstrated to efficiently prime influenza virus-specific CTLs *in vivo* (Deres *et al.*, 1989), making these conjugates attractive agents for the generation of fully synthetic vaccines. Due to its immune system modulating activity and the identification of the Toll like receptor 2 as its physiological receptor (Lien *et al.*, 1999; Aliprantis *et al.*, 1999), Pam3Cys-conjugates have seen a tremendous growth in interest within the last 5 years. Different derivatives of synthetic lipopeptides with central core of Pam3Cys moiety are using in the field of research namely Pam3Cys-SKKKK, Pam3Cys-OH, Pam3Cys-Ser(Glu)4-OH, Pam3Cys-SerSerAsn, and Pam3Cys-LGGGSKPK-NH2 etc.

IIIb) Pam3Cys (Pam3CSK4)
Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimicks the acylated amino terminus of bacterial lipoproteins. Pam3CysSerLys4 (Pam3CSK4) is a potent activator of the proinflamatory transcription factor NF-κB. Recognition of Pam3CSK4 is mediated by TLR2 which cooperates with TLR1through their cytoplasmic domain to induce the signalling cascade leading to the activation of NF-Kb (Fig. 2.22).

In the present study, effect of combination of Pam3Cys and miltefosine at sub-curative dose for the treatment of experimental visceral leishmaniasis in mouse model was studied.

IIIc) Pharmacological Class

**Chemical Name:**

N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4.Hydrochloride

**Molecular Formula:** C81H156N10O13S. 3HCl
Molecular Weight of Pam3CSK4 is 1509.6 - 109.5 g/Mol.

Fig.2.22 Chemical structure of Pam3CSK4
Source: www.pdbj.org

IIIId) Structural Characteristics and Mode of Action of Pam3Cys
Conserved patterns in diverse microbial molecules including lipoproteins, lipopeptides, lipopolysaccharide, flagellin, and nucleic acids are recognised by Toll-like receptor (TLR) family proteins and form complexes. These complexes play a crucial role in innate immunity (Akira & Takeda, 2004; West et al., 2006). Lipoprotein, lipopeptides, and peptidoglycan of Gram positive bacteria are recognized by TLR-2. TLR-2 forms a TLR-1/2 and or 2/6 heterodimer which recognizes triacyl and diacyl lipoproteins respectively (Takeuchi et al., 1999; Ozinsky et al., 2000; Takeuchi et al., 2000; Takeuchi et al., 2002). Pam3Cys is a synthetic triacyl lipoprotein and known activator of the TLR-2/1 heterodimer (Shimizu et al., 2008). These lipoproteins are anchored to the cell membrane via conserved N termini modified by lipid chains (Chambaud et al., 1999) and induce strong proinflammatory signals in macrophages (Henderson et al., 1996).

In the tri-acylated lipoproteins, the di-acylated glyceryl group is attached to the N-terminal cysteine via a thioether bond and the third lipid chain is connected to the cysteine via an amide bond. Palmitoyl groups are the most common lipid chains in the bacterial lipoproteins but other lipid chains are also found (Belisle et al., 1994; Braun, 1975; Mizuno, 1979; Zlotnick et al., 1988). Synthetic lipopeptide analogs containing di- or tri-acylated cysteine groups mimic the proinflammatory properties of lipoproteins, thus confirming that the acylated N-terminal cysteine is the principal immune stimulatory motif (Berg et al., 1994; Bessler et al., 1985; Seifert et al., 1990; Wiesmuller et al., 1992).
IIIe) Structural Characteristics of Pam3Cys -TLR-2/1 Complex

The crystallized complex contains a single Pam3Cys ligand shared by the TLR1 and TLR2 hybrids (Kim et al., 2007) (Fig.2.23). Two of the three lipid chains of the ligand interact with a pocket in TLR2, and the remaining amide-bound lipid chain is inserted into a narrow channel in TLR1. The TLR1 channel and TLR2 pocket are connected at the dimer interface, forming a long and continuous lipid-binding site. The overall shape of the complex resembles the letter “m” where the two C-terminal domains converge in the middle.

![Fig.2.23 Overall Structure of the TLR1-TLR2-Pam3Cys (Pam3CSK4) Complex](image)

The TLR1 fragments, the TLR2 fragments and the Variable Lymphocyte Receptors (VLR) fragments are shown schematically in green, blue, and gray, respectively. The central domains are colored in light green or light blue, and the Pam3CSK4 lipopeptide in red. Source: jin et al (2009); Cell

IIIif) Functions performed by Pam3Cys

The efficacy of treatment of Leishmania is compromised due to suppression of immune function during the course of infection. It is usually associated with a depression of Th1 cells and preferential expansion of Th2 cells. Pam3Cys, synthetic bacterial lipopeptide (bLP) and TLR-2/1 ligand is a potent activator proinflammatory transcription factor NF-κB (Aliprantis et al., 1999). It has also been used as adjuvants to activate cell-mediated immune responses. For exploring mechanisms Sieling et al (2003) stimulated human PBMCs with Pam3Cys and found that TLR2 ligand stimulate T cells to proliferate and
produce IFN-γ in an accessory cell-dependent manner and in the absence of exogenous protein antigens. It directly triggers the Th1 effector functions. According to Imanishi et al. (2007), in mouse Th1 cells, the stimulation by Pam3Cys (TLR2 ligand) directly induced IFN-γ production, cell proliferation, and cell survival without T cell receptor stimulation. Deetz et al. (2006) described the increased synthesis and secretion of IFN-γ by the dual stimulation with anti-TCR antibodies plus Pam3Cys. Brull et al. (2009) also reported the enhanced T-cell activity, as indicated by an increased proliferation and production of different cytokines such as IL-2 and IFN-γ by the stimulation of TLR2 ligand Pam3Cys. The remarkable study of Thoma-Uyszynski et al. (2000) described that pam3Cys triggers induction of IL-12 from human dendritic cells which further differentiate the naive T cells. The apoptotic signaling pathway is also activated by Toll-like receptor-2 (Aliprantis et al., 1999 Aliprantis et al., 2000). Several studies revealed that pam3Cys is also an efficient inducer of TNF-α from human monocytes (Siedlar et al., 2004; Crane-Godreau & Wira, 2005; Remer et al., 2006) which is a major player in anti-tumor immunity (Ruegg et al., 1998). Pam3Cys has also been previously reported as built-in immunoadjuvant (Buskas et al., 2006). Now a days, due to this property, Pam3Cys is very much in use against anticancer therapy because lipopeptide Pam3Cys, which is a TLR2 ligand, has been attached to tumor-associated carbohydrate antigens which is used in the assembly of synthetic carbohydrate-based vaccines (Buskas et al., 2009; Renaudet et al., 2010). Derivatives of Pam3Cys were also labelled with fluorescence markers to investigate the mechanism of action of these synthetic vaccines (Weterings et al., 2009).

**IIIg) Application of Pam3Cys in the Field of Leishmaniasis**

A straight-forward synthetic access to lipopeptides based on standard solid-phase synthesis protocols makes these lipoconjugates very attractive agents for various biological applications.

For parasitic disease leishmaniasis, synthetic immunostimulator Pam3Cys as conjugated to the synthetic cap of tetra saccharide to create fully synthetic carbohydrate vaccine (Hewitt & Seeberger, 2001). In another study, functional capacity of CD8 cells of DCL patients was restored by pre incubating them with TLR2 agonists: Pam3Cys. This is the first report showing that stimulation of the TLR2 can restore effector mechanisms antigen-specific
proliferation and IFN-γ production in functionally exhausted CD8 cells from patients with diffuse cutaneous leishmaniasis (Hernández-Ruiz et al., 2010). The involvement of TLR2 in cytokine and reactive oxygen species (ROS) production by PBMCs in response to L. major phosphoglycans (PGs) was also reported (Kavoosi et al., 2009).

2.6.2 Therapeutic Switching or “Piggy Back Chemotherapy”

A) Introduction

Therapeutic Switching (also known as Drug repurposing, Drug re-profiling, Drug repositioning and Drug re-tasking) is the application of known drugs and compounds to new indications (i.e., new diseases). It is not a new idea. It has been happening since early 1990s, mostly as a unanticipated process and growing in importance in the last few years, as an increasing number of drug development and pharmaceutical companies see their drug pipelines drying up and realize that many previously promising technologies have failed to deliver ‘as advertised’. Using therapeutic switching, pharmaceutical companies have achieved number of successes, for example Pfizer's Viagra in erectile dysfunction and Celgene's thalidomide in severe erythema nodosum leprosum. Smaller companies, including Ore Phamaceuticals, Biovista, Numedicus and Melior Discovery are also performing therapeutic switching on a systematic basis. These companies use a combination of approaches including in silico biology and in vivo/in vitro experimentation to assess a compound and develop and confirm hypotheses concerning its usage for new indications.

B) Need of Therapeutic Switching

Drug repositioning has been attracting the interest of pharmaceutical and biotech companies for two reasons:

(i) Commercial reason: On the commercial side, the simple truth is that company pipelines are not growing at the desired pace. At the same time, the return per dollar spent in the lab has been steadily decreasing. Pharmaceuticals have been trying a number of tactics such as ‘light reformulations’ that extend patent protection and company buyouts that instantly grow their pipelines with new compounds. These tactics however are proving less viable, as regulatory bodies tighten their criteria and we begin to run out of companies to buy.
(ii) Scientific reason: On the scientific side, the promise of certain technologies is not unfolding as hoped for. At the same time we are all beginning to realise some of the limitations of our existing knowledge, not only at the disease, but also at the biology and drug mechanism levels. In this context, it makes sense to ‘milk the knowledge cow’ by recombining our existing knowledge in as many new ways as possible, with the specific aim of identifying workable drug-therapeutic area correlations that have so far escaped our attention and screening systems.

C) Therapeutic Switching used in Leishmanial Chemotherapy

Leishmania infection is strongly linked with poverty. Risk for infection is mediated through poor housing conditions, environmental sanitation and lack of personal protective measures. Since, poverty is associated with poor nutrition and other infectious diseases; the risk that a person will progress to the more serious stage of infection is very high. Lack of access to healthcare causes delays in appropriate diagnosis & treatment and increases leishmaniasis morbidity & mortality (Homsi & Makdisi 2010).

Treatment of both visceral leishmaniasis and cutaneous leishmaniasis for the past 50 years has been dependent upon the pentavalent antimonials sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). In recent decades there has been evidence of a decrease in parasite sensitivity to these drugs in the treatment of VL. Now in Bihar State, India, over 60% of cases of VL caused by L. donovani are not responding to treatment with antimonials. Antimonials are less effective against CL and mucocutaneous leishmaniasis, possibly because of poor pharmacokinetics and the variation in species sensitivity (there are at least 12 different species of Leishmania that cause CL). Two antibiotics, amphotericin B and paromomycin, have re-emerged as important antileishmanials following the development of improved formulations.

Paromomycin also known as aminoglycoside antibiotic, aminosidine or monomycin). In bacteria, it inhibits protein synthesis by binding to 30S subunit ribosomes, causing misreading and premature termination of mRNA translation. In Leishmania, paromomycin also affects mitochondrion (El-On et al., 1992). Paromomycin has become a useful antileishmanial for CL, through the development of topical formulations. Additionally, a parenteral formulation of paromomycin has proved to be an effective treatment for VL.
Whereas amphotericin B is a fungal antibiotic. It makes complexes with 24-substituted sterols, such as ergosterol in cell membrane, thus causing pores which alter ion balance and result in cell death (Roberts et al., 2003). Leishmania parasites having same sterol (ergosterol) in their cell wall. The efficacy of the antibiotic amphotericin B has been improved by the development of less toxic lipid formulations (L-ampB). Although originally developed for the treatment of systemic mycoses, L-ampBs have been successfully exploited for VL with the additional advantage of targeting the drug to infected macrophages of the liver and spleen. Three commercial L-ampB formulations have been used for the treatment of VL; of these, the unilamellar liposome formulation AmBisome has proved to be most effective against VL in immunocompetent adults and children in Europe, Sudan, Kenya and India (Berman et al., 1998). Oral amphotericin B is currently being assessed in Phase I as well as undergoing (animal) efficacy studies for VL (den Boer et al., 2009).

Azoles are the one of the others that were also developed as antifungal drugs. Leishmania resemble fungi in synthesizing 24-substituted sterols such as ergosterol. Azoles, such as ketoconazole, inhibit 14α-demethylase, a key enzyme in this sterol biosynthesis pathway. Ketoconazole, itraconazole and fluconazole have undergone several trials for CL and VL with equivocal results. In one controlled trial, ketoconazole was found to have some activity against L. mexicana, but not against L. braziliensis infections (Navin et al., 1992). Some recent encouragement has been given by the oral activity of posoconazole in a Leishmania amazonensis experimental model (Al-Abdely et al., 1999).

Bisphophonates, for example, risedronate and pamidronate, which are in widespread use in the treatment of bone disorders such as osteoporosis, have also shown activity against leishmaniasis in experimental models (Rodriguez et al., 2002; Yardley et al., 2002). These studies followed the characterization of acidocalcisomes in trypanosomatids with high polyphosphate and pyrophosphate content because there is a hypothesis that bisphophonates could interfere with pyrophosphate metabolism, although it is now thought that the prime target might be farnesyl pyrophosphate synthase – a key enzyme in isoprenoid biosynthesis (Martin et al., 2001).
Two other oral drugs are in trials for the treatment of VL: the 8-aminoquinoline sitamaquine (originally WR6026) and the anticancer drug miltefosine (Berman, 2005). Drug mechanism of sitamaquine for leishmaniasis is unknown. It might affect mitochondrial electron transport chain (Yeates, 2002). Sitamaquine has undergone Phase II trials but issues remain about safety and relatively limited efficacy (under 90%). Even with an optimistic clinical development scenario, it is unlikely that it will be registered for VL before 2014 (den Boer et al., 2009).

Miltefosine (hexadeclyphosphocholine, Impavido®) is an alkylphosphocholine that was originally developed as an anticancer agent in the early 1980s. In the mid-1980s, its potential activity for VL was identified and registration was achieved in India in 2003 (den Boer et al., 2009). The primary effect of miltefosine in leishmania is uncertain but possible mode of inhibition can be ether remodelling, phosphatidylcholine biosynthesis, signal transduction and calcium homeostasis (Croft et al., 2003).

Leishmania is an obligate parasite of macrophages thus immunotherapeutic approaches to activate the host cells to kill the intracellular amastigotes have been pursued. Interferon γ (originally developed as antiviral agent {Fensterl & Sen, 2009}), used in combination with antimonials, was effective in cases of VL in Brazil and India. In the Mediterranean and Brazil, canine leishmaniasis caused by *L. infantum* is a problem, but there are no effective drugs for this condition (Murray, 2001).

Current research for VL has mainly been focused on the biological aspects of the parasite, an approach that is not of benefit to the many patients who need treatment today and not targeted to identifying promising new drugs. However, with the foundation of iOWH and DNDi, prospects have been improving. To register one new drug through new formulations of existing treatments and therapeutic switching is one of the long term objectives of DNDi. Oral amphotericin B is currently being assessed in Phase I as well as undergoing (animal) efficacy studies for VL and another oral formulation of amphotericin B (iCo) is currently in preclinical development. Sitamaquine (8-Aminoquinolone; analog of the antimalarial agent primaquine) has undergone Phase II trials but issues remain about safety and relatively limited efficacy (under 90%) (http://www.dndi.org/overview-dndi/objectives.html).
Drug screening programs for VL have received a financial boost; three highly effective new treatments for VL were identified through therapeutic switching (amphotericin-B, paromomycin and miltefosine) and have been licensed in the past 10 years. This approach has been one of the most important methods for the study and clinical introduction of novel drugs for leishmaniasis and can deliver new drugs more quickly and at lower cost as much of the development work has already been done. On reviewing the available treatments against VL, it is concluded that therapeutic switching is an efficient strategy for the therapy. It may provide new therapeutic regimen in the future also.

2.6.2.1 Antifungal Drugs used in Present Study

Two antifungal agents were used in this study with the combination of standard antileishmanial agent, miltefosine namely fluconazole and ketoconazole. Fluconazole is classified as triazole and ketoconazole comes under imidazole class. Detailed description of both the drugs has already been described earlier.