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Review

Biological targeting and drug delivery in control of Leishmaniasis

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Leishmaniasis is a neglected tropical disease caused by a protozoan parasite of the genus *Leishmania*. Visceral leishmaniasis is the most severe type and is transmitted by the phlebotomine sandflies of genera *Lutzomyia* (New World) or *Phlebotomus* (Old World) to human and other vertebrates. Leishmaniasis is widespread in developing countries with current mortality rate of 50 thousand deaths per year. The parasites adopt different biochemical approaches to evade the host immune system. Knowledge in chemical control of leishmaniasis is currently emerging and not many drugs are available. Control of parasite is complex and WHO has put an ardent appeal for development of drugs and delivery devices against leishmaniasis. Main-stay in treatment of leishmaniasis is pentavalent antimonials but second-line drugs like amphotericin B and pentamidine are available. Clinical acceptability of drugs is poor due to severe toxicity, poor bioavailability, improper localization and recent appearance of resistant variants. Interest in leishmanicidal chemotherapy is therefore renewed and biochemical strategies or improved delivery appear to be a solution. Trends in control of leishmaniasis also include specific applications of low-cost, locally available plant drugs in different delivery devices. This work attempts to present a comprehensive overview of the different approaches to targeted leishmanicidal chemotherapy.

Key words: Leishmaniasis, host-immune system, pentavalent antimonials, delivery devices, polymeric nanoparticles.

INTRODUCTION

Leishmaniasis is a rapidly spreading parasitic disease, and presently a major cause of morbidity and mortality in the developing world. Leishmanial parasitic infections lead to a number of clinical conditions, of which, visceral leishmaniasis (VL) is the most prevalent. VL is fatal if untreated and the World Health Organization has classified it as one of the top ten threatening infective conditions (WHO, 2001; 2010). An estimated 350 million people are at risk of infection with an average of 2 million new cases reported annually including about 50 thousand deaths due to VL (WHO, 2010). Leishmaniasis is also spreading as an HIV-associated infection with increasing appearance of drug resistant types (Guerin et al., 2002). HIV infection can increase the risk of VL development by about 100 fold in endemic areas (Daher et al., 2009). Different forms of infections include cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and post-kala-azar dermal leishmaniasis (PKDL). Clinical characteristics of VL include high fever, hepatomegaly and splenomegaly, jaundice, anemia and weight loss. Diarrhea and cough are also common. Hypoalbuminaemia and polyclonal-hypergammaglobulinaemia (IgG and IgM) constantly appear. Hyperpigmentation of the skin is common in VL and thus giving an alternate name - kalazar (Black fever) (Awasthi et al., 2004).

The geographical distributions of leishmaniasis relate to the growth of sandfly vectors which are particularly dominant in the tropical and temperate regions. The leishmaniases is considered endemic in 88 countries (16 least developed countries and 72 developing countries). While ninety percent of cases with cutaneous forms of leishmaniasis occur in Afghanistan, Algeria, Brazil, Iran,

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Peru, Saudi Arabia and Syria, ninety per cent of VL cases are found in Bangladesh, Brazil, India, Nepal and Sudan (WHO, 2001). Leishmaniasis is also reported in Europe as a result of Leishmania-HIV co-infection occurring mainly in Italy, France, Romania, Switzerland and Germany (Desjeux et al., 2000). Old World species of *Leishmania*, like *L. donovani* and *L. major*, are prevalent in southern Europe to Africa, the Middle East, and throughout southern Asia. The New World species like, *L. mexicana*, *L. amazonensis*, and *L. chagasi* are common throughout the South and Central America and as far north as the southern states of the United States. *Leishmania chagasi*, is most prevalent in South America and it induces DNA damage in the peripheral blood and spleen cells (Oliveira et al., 2011).

Management of Leishmaniasis, is more complicated compared to other parasitic infections, as the parasite can rapidly invade macrophages and differentiate into a proliferative form called amastigotes. This ultimately destroys the host mononuclear phagocytic system making it vulnerable to many other common infections. Successful invasion of the parasite into host immune system and survival are linked to expression of specialized and stage specific molecules (Turco and Descoteaux, 1992), alterations in macrophage membrane permeability (Quintana et al., 2010) and electrical activity caused by the parasitic infection (Camacho et al., 2008). Molecular specificity therefore is necessary for targeting therapeutic agents to the leishmanial parasite after its successful invasion into the host. This review is an overview on the problem of leishmaniasis and the current treatment strategies being adopted to mitigate this disease. We have searched Pubmed, Scopus and ACS databases with the key words leishmaniasis, treatment, antimonials, liposomal amphotericin B from 1985 to 2011.

Life cycle of *Leishmania* parasite

A basic understanding of parasite life cycle through different stages is helpful in devising specific strategies for treatment. Different species of the sandfly (genus *Phlebotomus* or *Lutzomya*), are believed to be vectors. A need for protein during the egg laying period make the female sandfly take a blood meal (www.who.int), and thus transmit the leishmanial parasite to humans and domestic animals.

During its life-cycle (Figure 1), the protozoan parasite of the genus *Leishmania* alternates between two forms: the amastigote (replicative) form and the promastigote (flagellar) form. Amastigotes reside in phagolysosomal compartments of macrophages in humans and other vertebrate hosts and promastigotes are found in the midgut of the sandfly vector. When the amastigote-infected blood of the vertebrate host is sucked by the sandfly, transformation of amastigote to promastigote form starts within hours of ingestion in the insect gut. Within 24 to 48 h, the amastigotes are transformed into actively motile promastigotes which divide by binary division. Between 6 to 9 days after an infective meal, the promastigotes migrate to the sandfly midgut and when the sandfly bites a new host the promastigotes are transferred. After a bite there is rapid infiltration of neutrophils and macrophages into the bite site which engulf the promastigotes. Here the promastigotes become immotile and transform again into amastigote form. This phenomenon has also been observed in imaging-based studies conducted *in vitro* (Beattie et al., 2011). The amastigotes invade and reside in the cells of reticuloendothelial (RE) system where they multiply by binary fission. As many as 50 to 200 amastigotes get liberated because of cell rupture and failure of the immune response mainly due to impaired T helper cell type 1(Th1) response (Murray et al., 1989). The parasites are subsequently liberated into the circulation to invade fresh cells.

Currently used drugs in the treatment of Leishmaniasis

Current treatment (Table 1) involves use of pentavalent antimonials (SbV) like sodium stibogluconate (Pentostan), *N*-methylglucamine (Glucantime), amphotericin B and pentamidine (Murray et al., 2001). Among these sodium stibogluconate and meglumine antimoniate is used as first-line chemotherapeutic agents against all forms of leishmaniasis including visceral. Although, SbV drugs have long been used, information regarding their chemistry and mode of action is limited. Drug resistance is a major limiting factor and antimonial-resistant parasites isolates are amply reported (Grogl et al., 1992). Additionally, long term administration and higher doses give rise to toxic effects like increased levels of various marker enzymes such as elevated creatine phosphokinase and alkaline phosphatase levels (Oliveira et al., 2011), hepatomegaly and typical skin reactions for heavy metals. SbV resistance development in *L. donovani* is due to efflux of antimonials from parasite infected host cells via up-regulation of P-glycoprotein (P-gp,MDR1) and expression of multidrug resistance-related protein 1 (MRP1) (Basu et al., 2008).

Pentamidines have been used in leishmaniasis since 1939 and are effective against kala-azar (Monzote, 2009). Because of their toxicity and potential side effects they are used as drugs of second choice (Thakur et al., 1984; Fusai et al., 1995; Chakraborti et al., 1997; Berman et al., 1999). Pentamidines bind to tRNA through non-specific hydrophobic interactions and inhibits aminoacylation and translation of the replicating parasite (Sun et al., 2008). Adverse reactions of the injectable form of pentamidine include hypotension, hypoglycemia, leucopenia, thrombocytopenia, cardiac arrhythmia, acute renal failure, elevated serum creatinine level, nausea, and fever (Monzote, 2009).

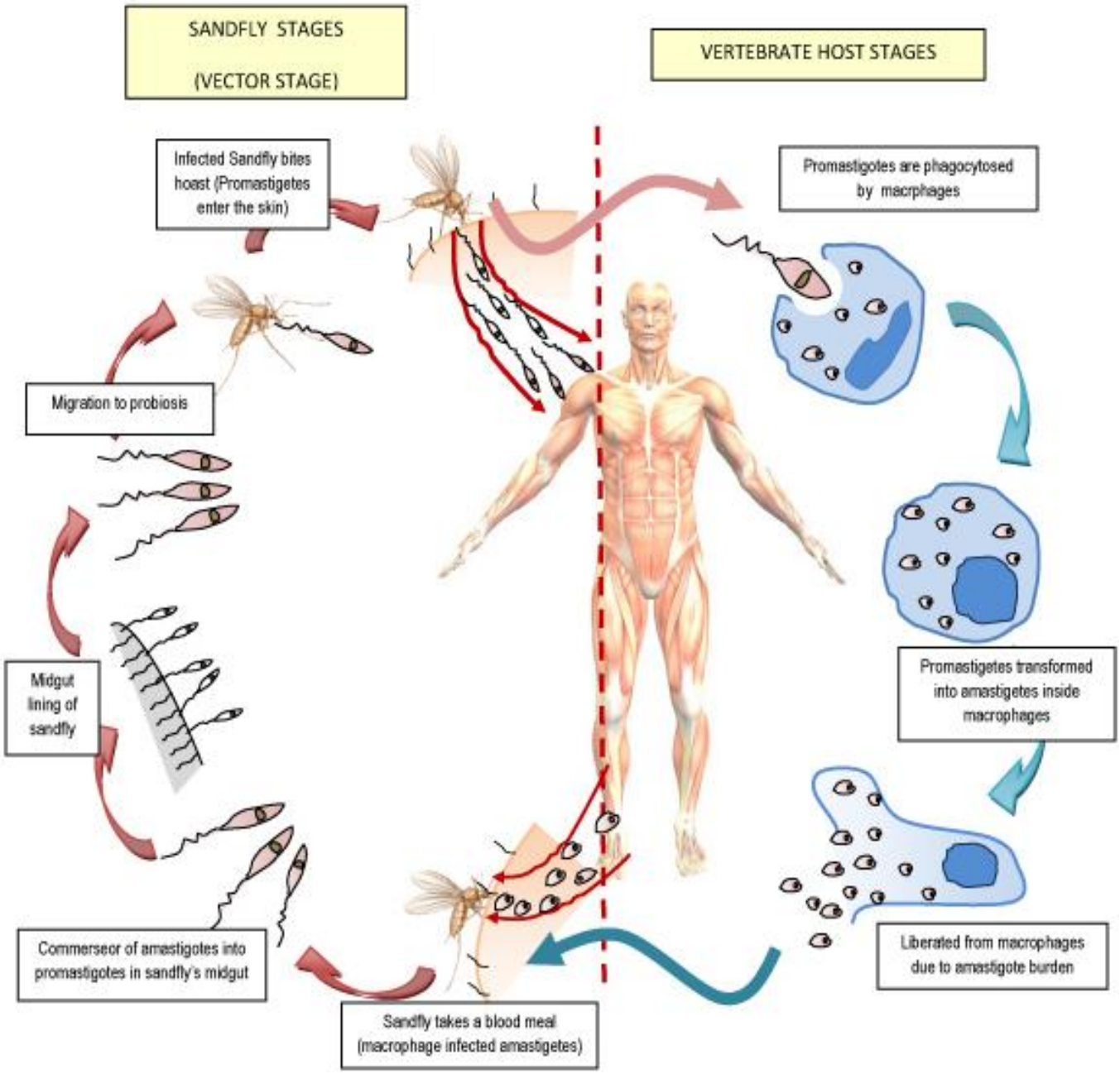
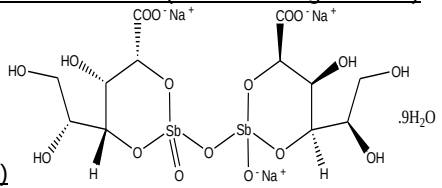
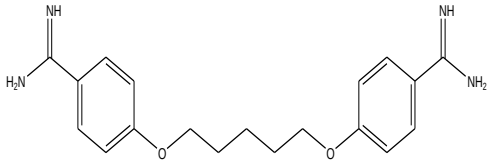
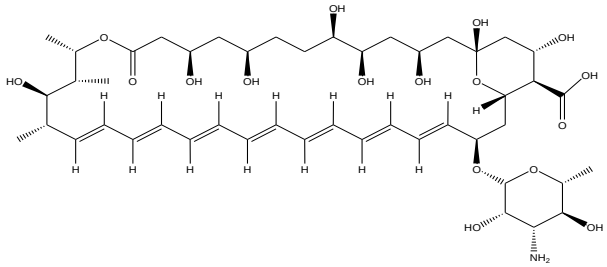
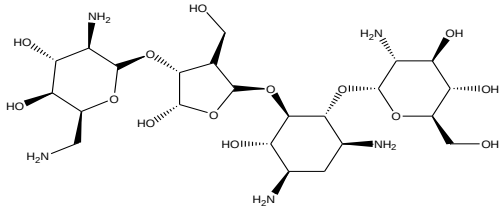
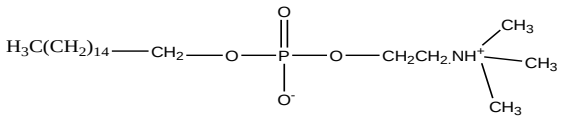


Figure 1. Life-cycle of *Leishmania* Parasite.

Amphotericin-B, (AmB) a macrolide antibiotic produced by *Streptomyces nodosus* is a very successful but highly toxic in leishmanial therapy. AmB affinity for the ergosterols of parasitic cell membranes accounts mostly for its lethality. The fundamental mechanism is assumed to be formation of an intimate binary complex of AmB with membrane sterols. The association evokes changes in membrane permeability through uncontrolled loss of ions from the cells due to formation of barrel shaped transmembrane pores which results in cell lysis (Cohen et al., 1987). Membrane permeability is also influenced by AmB-induced lipid peroxidation of cell membranes resulting in fragility (Brajtburg et al., 1985). AmB inhibits membrane enzyme- H^+-ATPase in fungal cells (Brajtburg et al., 1996) and Na^+/ K^+-ATPase in mammalian cells (Vertut-Do et al., 1988). This inhibition can be a cause for loss in proliferative ability by depletion of cellular energy reserves (Schindler et al., 1993). However, AmB shows poor gastrointestinal absorption and negligible bioavailability due to the hydrophobicity of the polyene structure (Gershkovich et al., 2009). Also, it can interact with the mammalian cell membrane causing cellular dysfunction

Table 1. Antileishmanial drugs and their mode of action.

Generic name of the drug	Mechanism of action	Limitation
1. Pentavalent antimonials (sodium stibogluconate)  (Pentostam)	Action on the macrophage, Activated within the amastigote form	Limited information regarding chemistry and mode of action: cardio toxicity, renal insufficiency, pancreatitis, anemia, leucopenia headache, nausea, vomiting, abdominal pain on long-term administration.
2. Pentamidines [Dimedene analogs such as mepacrine, pentamidine isethionate (Pentam-300)] 	Binds to tRNA and inhibits aminoacylation and translation of the replicating parasite.	Emergence of drug resistance especially in HIV co-infections. Adverse reactions of injectable form of pentamidine: hypotension, hypoglycemia, leucopenia, thrombocytopenia, cardiac arrhythmia, acute renal failure, elevated serum creatinine level, nausea, fever.
3. Amphotericin B (Polyene antibiotics) 	Binds with the ergosterols of the parasitic cell membranes thus forming a binary complex with the membrane sterols resulting in pores which causes changes in membrane permeability and ionic balance leading to parasitic cell death	Poor gastro-intestinal absorption and negligible bioavailability. Also may react with mammalian cell membrane causing cellular dysfunction.
4. Paromomycin (an aminocyclitol-aminoglycoside antibiotic) 	Impairs the macromolecular synthesis and alters the membrane properties of leishmania	Mainly used in the cutaneous form of the disease Has limited use in the treatment of visceral leishmaniasis.
5. Miltefosine 	Mechanism of action uncertain, possible inhibition by phosphatidylcholine biosynthesis, signal transduction and regulation of calcium homeostasis	Development of quick drug resistance

(Bolard et al., 1991). The commonly used formulation of deoxycholate complexed AmB micelles (Fungizone), is highly toxic to patients, often causing decreased renal function, anaphylaxis, chills, high fever, nausea, phlebitis, anorexia and other adverse effects. These adverse reactions coupled with long therapeutic regimes limits its usefulness as in anti-infective therapy in general (Hartsel et al., 1996). Lipid formulations of AmB reduce toxicity to non-target tissues but development of resistance cannot be disregarded (Croft et al., 2006)

Paromomycin, an aminocyclitol-aminoglycoside antibiotic was originally used for treatment of bacterial infections since the 1960s (Monzote, 2009). Combination therapy using amphotericin B or its liposomal formulation along with miltefosine or paromomycin has shown promising anti-leishmanial activity (Griensven et al., 2010). Paromomycin acts by impairing the macromolecular synthesis and altering the membrane properties of Leishmania (Monzote, 2009). A randomized, controlled, phase-3 open-label study comparing injectable paromomycin against AmB was conducted in Bihar, India and it was inferred that it was non-inferior to AmB alone (Sundar et al., 2007). Paromomycin in combination with either cycloheximide or chloramphenicol interferes with the dissociation of mitochondrial and cytoplasmic ribosomes thereby inhibiting protein synthesis (Sundar et al., 2008). Alternative chemotherapy choices in leishmaniasis are otherwise limited, and some other compounds used include miltefosine, atovaquone, allopurinol, ketoconazole, aminosidine and Imiquimod. (Olivier et al., 1998; Buates et al., 1999).

Most of these drugs suffer from multifarious limitations and run the risk of development of drug resistance. Thus, effective, convenient, low toxic and low-cost chemotherapy is necessary. Incomplete treatment schedules and patient noncompliance are other limitations which increase the occurrence of drug resistant variants. Development of highly specific and sensitive non-invasive diagnostic tools could be the newer weapons to combat the spread of leishmaniasis (Guerin et al., 2002).

Different plant-derived natural compounds (Figure 2) are presently experimental antileishmanial chemotherapies. More than twenty plants were explored containing specific molecules with anti-leishmanial activity. Benzylisoquinolines, β-carboline alkaloids, iridoid and steroidal glycosides, terpenoids, flavonoids and other metabolites such as acetogenin, aregentilactone are used in anti-leishmanial therapy (Gupta et al., 2010). The antileishmanial activity of ethanolic extract of *Artemisia indica* leaves containing artemisin was demonstrated against several *Leishmania* species (Sen et al., 2010). Azole based antileishmanial agents derived from plants were also screened. Al-Qahtani et al. (2009) reported *in vitro* activity of 44 derivatives of 1,3,4-thiadiazole against promastigote forms of *L. donovani* in micromolar levels and thiophenyl azoles (Marrapu et al., 2011). Nicotinamides, exert *in vitro* antileishmanial activity by improving the antileishmanial activity of trivalent antimony in a synergistic manner and exhibit additive effects in combination with amphotericin (Gazanion et al., 2011).

Chemotherapeutic agents have solubility limitations and low bioavailability, necessitating high doses for effective chemotherapy. Combination therapy of natural products along side antibiotics or synthetic drugs is also an emerging trend in management of leishmaniasis therapy (Tiuman et al., 2011).

Antileishmanial vaccines

In order to overcome parasite drug resistance, vaccination as an alterative to chemotherapy has been studied but the efficacies are uncertain and applications not yet wide spread (Reithinger et al., 2007). Identification of leishmanial surface molecules and killed parasites components were assayed for vaccination. Live-non-attenuated vaccines are the most primitive approach for 'Leishmanization'- generating immunity to leishmania (Khamesipour et al., 2006). Genetic alteration of *Leishmania* parasite, maintaining its immunogenicity but destroying its virulence, is a current strategy in leishmanial vaccine development. Gene replacement through homologous recombination was later followed due to risks in virulence reversal (Capecchi et al., 1989). The first vaccine developed was dihydrofolate reductase thymidylate synthase attenuated parasites experimented successfully in mouse models (Titus et al., 1995) but failed in higher primates (Amaral et al., 2002). The vaccine efficacy of soluble leishmanial antigens (SLA) from *Leishmania donovani* promastigote membrane entrapped in liposomes was studied for immunotherapy of VL (Bhowmick et al., 2007). The immune response was primarily mediated through T cells with a surge in Th cell cytokines. Simple and cheaper production, sufficient temperature stability and easier storage requirements of DNA vaccines makes them most appealing among leishmanial vaccines (Handman, 2001). LJM11 is an abundant salivary protein from the sand fly *Lutzomyia longipalpis*. Mice immunized with plasmids coding for the *L. longiplapis* salivary proteins showed protective immunity against *Leishmania major* infection. Immunization with *Lu. longipalpis* saliva or with LJM19 DNA plasmid induced a Delayed-Type Hypersensitivity (DTH) response following exposure to *L. longipalpis* saliva. Also, splenocytes of exposed mice produce IFN-γ upon stimulation with LJM11 which leads to induction of T-helper cell function. These findings suggest the possibility to develop a vaccine using a single component of *Lu. longipalpis* saliva to generate protection against different species of *Leishmania*, even those transmitted by a different vector (Xu et al., 2011). However, more investigation is necessary before vaccination can be proved to provide complete protection against leishmaniasis. Other azoles used include substituted benzyloxy furanyl.

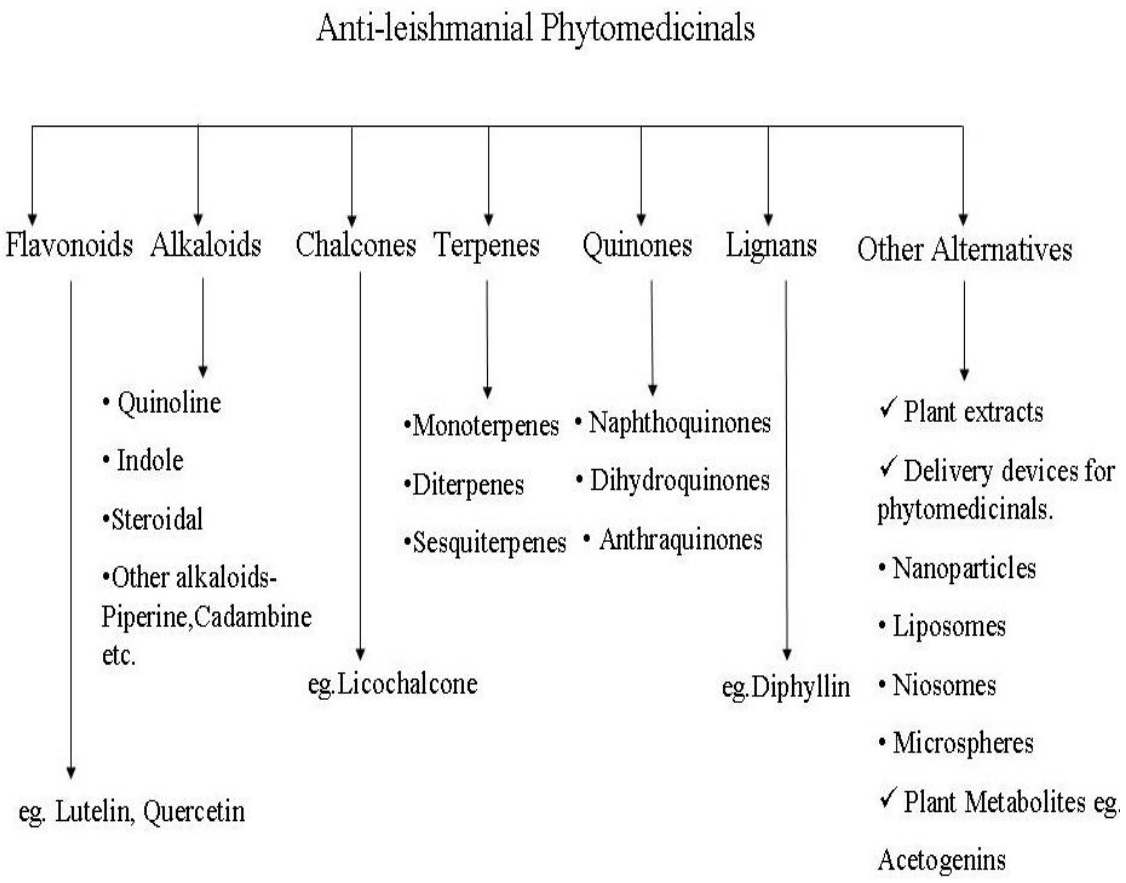


Figure 2. Classification of local available antileishmanial plant drugs.

Host-parasite interactions, evasion of host immune system and survival

Despite development of treatment strategies against leishmaniasis, it is still widespread mainly because the parasite manages to survive within the host by evading the host defense system, adopting various mechanisms acquired through years of evolution. *Leishmanial* parasites interact with the host defense at molecular levels (Schmid-Hempel, 2009). Transmission and epidemiology of leishmaniasis are dependent on feeding habit of specific vector, host genetics and successful inhibition of host defensive oxidative pathways. Some of the biochemical mechanisms include inhibition of phagolysosome formation, abnormal activation of protein kinase C and scavenging of the reactive oxygen species (Villa et al., 2002; Olivier et al., 2005,). Survival strategies also include prevention of apoptosis of infected macrophages, impairment of macrophage antigen presenting function by MHC molecules and impairment of responsiveness to cytokines (Bogdan and Röllinghoff, 1999). Additionally, the parasite interferes in complement activation and humoral immunity mechanisms by blocking the protective T helper cell response within the host's body (Villa et al., 2002).

Role of parasite surface and secreted molecules

During their life cycle *Leishmania* parasites are faced with hostile environments from the gut of the sandfly where digestive enzymes are abundant to the hydrolase-rich phagolysosomes of host macrophages. The mechanisms allowing this pathogen to survive and proliferate in these hostile conditions involve the expression of stage-specific virulence determinants including the lipophosphoglycan (LPG), the major cell surface glycoconjugate of promastigotes (Chang et al., 1990).

Lipophosphoglycans (LPG)

The dominant cell surface molecule of promastigotes is lipophosphoglycan (LPG). LPG is a part of glycosylinositolphospholipid (GPI)-anchored polymer with multiple repeating disaccharide– phosphate units, [Gal (β1, 4) Man→(α 1- PO_4 6)] (between 16 to 30 units), glycan side chains and a capping oligosaccharide (Kaye et al., 2011). The repeating units of LPG, is essential for the interaction of promastigotes with both the insect vector and the mammalian host. In mutant parasites lacking the LPG these parasites were rapidly

destroyed by phagocytosis because parasite host cell interaction was greatly affected (Handman et al., 1986; Naderer et al., 2008).

During the initial stages of macrophage infection LPG promotes intracellular survival of promastigotes by inhibiting the fusion of the parasite-containing phagosome with the lysosomes. Inhibition of phagosome–endosome fusion is an intramacrophage survival strategy adopted by a variety of intracellular pathogens. Surface components of LPG cause alteration of fusion properties of the endocytic system (Miao et al., 1995; Desjardins and Descoteaux 1998). Thus, LPG has a natural role of in protecting *Leishmania* parasites from digestion in host lysosomes immediately upon invasion. In the parasitophorous vacoule of macrophages the survival of promastigote is dependent on the LPG repeating units which also scavenge the reactive oxygen species generated during respiratory bursts inside the macrophage cells during phagocytosis because of their unique structure of repetitive oxidizable phosphorylated disaccharide units (Desjardins and Descoteaux 1997). The generation of reactive oxygen intermediates (O_2 and H_2O_2) by the NADPH oxidase system are recognized as control mechanisms for *Leishmania* spp (Bogdan and Röllinghoff, 1999). The action of NADPH oxidase is dependent on the protein-kinase C (PKC) activation. LPG and gp63 (the major surface protease for *Leishmania*) contribute to abnormal activation of PKC and reduction of its translocation to the membrane. Additionally, several million copies of LPG at the promastigote surface form a dense glycocalyx that can provide a physical barrier against the action of hydrolytic enzymes. LPG also acts as the primary ligand for multiple macrophage recognition receptors (Naderer et al., 2004). LPG reduces the phagocytic capacity of macrophages and it causes exclusion of synaptotagmin V, an exocytosis regulator from the nascent phagosome which is responsible for increased survival and could lead to greater overall parasite fitness (Vinet et al., 2011).

Other surface proteins

In addition, gp63 (promastigote surface protease) is a surface glycoprotein (zinc-dependent metalloprotease) found throughout the promastigote surface but is less abundant than LPG (Olivier et al., 2005). Like LPG, it plays an important role in amastigote survival and modulation of the host response by inhibiting degradative phagolysosomal enzymes. It also blocks the oxidative burst through abnormal activation of protein kinase C, prevents apoptosis and antigen presentation by the MHC molecules (Giorgione et al*.,* 1996). Contreras et al. (2010) showed that *Leishmania* parasites use their most abundant surface protein GP63 to inactivate the transcription factor: Activated Protein-1(AP-1) which is involved in transcription of genes coding for antimicrobial functions of macrophages. Another mechanism for survival is cleavage-dependent activation of macrophage protein tyrosine phosphatase (PTPs) which internalizes GP63 responsible for *in vivo* progression of disease (Gomez et al., 2009).

The most abundant promastigote surface molecule is glycosylinositol phospholipid (GIPL), a class of GPI-linked glycolipids synthesized by the parasite (Olivier et al., 2005). Though the exact function is unclear it is presumed that this protects the promastigote from lysosomal hydrolases and minimizes the release of peptides from the parasite that could be presented to the host immune system by MHC class II proteins (Naderer et al., 2008). These structural components on the surface of the parasite coupled with other sophisticated mechanisms (Olivier et al., 2005) help it to establish a successful host-parasite relationship and subvert the immune response.

Immune-suppressive effects of *L. donovani*

Leishmania parasite evades both the innate and adaptive immune responses to survive within the host cell. The parasite inactivates the immune cells or the signaling pathways thereby facilitating its survival within the host cell (Olivier et al., 2005). The leishmanial parasite inhibits the complement cascade by degrading host proteins and by active interference of signaling compounds (Nunes et al., 1997). Parasites adapt even to feed on host immune molecules and the cell cytokines were used as the parasitic growth factors (Damian et al., 1997). Signaling pathways are vital in the functioning of immune system. The *leishmania* parasite inhibits interleukins IL-12 in dendritic cells and macrophages and induces IL-10 to avoid clearance (Locksley et al., 1993; Sacks et al., 2002).

Evasion of the host complement system

The parasites protect themselves against complement lysis by shedding of the lytic membrane complex (C5b-C9) with spontaneous release of C5b-C9-complexes from the parasite surface. This may be attributed to the elongation of the phosphoglycan chain of the LPG molecule on the surface (Villa et al., 2002). In addition, the leishmanial protein kinases phosphorylate several components of the complement activation system with subsequent inhibition of the classical and alternative complement pathway (Hermoso et al., 1991). The gp63 metalloproteinase is also involved in the resistance to complement–mediated lysis (Brittingham et al., 1995).

Evasion of the cell mediated and humoral response

Studies performed on mice infected with *Leishmania major* demonstrated that host defense against this infection depends on the interleukin-12 (IL-12) driven

expansion of the T-helper 1 cell subset, with production of cytokines such as interferon-gamma (INF-γ), which activate macrophages for parasite killing through the release of nitric oxide in the early stages of infection (Bogdan and Röllinghoff, 1996; Carrera et al., 1996). Secondly, the parasite inhibits antigen presentation by MHC molecules. *L. donovani* amastigotes interfered with the upregulation of the MHC Class II molecules by the IFN-γ at the transcription level. Alternatively, *Leishmania* could down regulate MHC class II expression also by a posttranslational mechanism (Souza-Leao et al., 1995). The parasites also inhibit macrophage apoptosis thus promoting its survival within the macrophage. Additionally, gp63 from *L. major* and *L. donovani* cleave CD4 molecules on T cells, interfering with the stabilization of the interaction between antigen-presenting cells and T helper cells (Locksley et al., 1993). Inhibition of IFN-γ production results in a survival advantage to the parasite (Bogdan et al., 1999). In addition, attachment and penetration of *L.donovani* promastigotes and their subsequent conversion to amastigotes within macrophages failed to induce IL-1 synthesis. Reiner et al. (1987) observed that *L. donovani* has the ability to both evade and suppress the macrophage IL-1 response which results in inhibition of T-cell activation and defects in cell-mediated immunity. Additionally, the parasites induce the development of the cytokines TGF-β and IL-10 which counteract the development of T-helper cells and inhibit killing of the *Leishmania* species. The parasite modulates the host cell signaling after infection leading to the inhibition of macrophage functions.

Manipulation of the signaling pathways of the host macrophage

Leishmania internalization within the macrophage is a receptor-mediated event, and this initial host-pathogen interaction is responsible for a rapid activation and deactivation of several signaling pathways in macrophage functions like phagocytosis, chemokine secretion, and prostaglandin secretion. The parasite has evolved strategies to interfere with a broad range of signaling processes in macrophage that includes Protein Kinase C, the JAK2/STAT1 cascade, and the MAP Kinase pathway (Olivier et al., 2005; Shadab et al., 2011).

Mitogen-activated protein-kinases (MAPKs) constitute one of the important intracellular signaling pathways in eukaryotic cells like macrophages which regulate their accessory and effector functions including production of proinflammatory cytokines and NO. *L. donovani* infection of macrophage leads to the alteration of MAP Kinase pathway, which in turn promotes parasite survival and propagation within the host cell. *Leishmania* can also activate various molecules that inhibit intracellular signaling cascades. An important negative regulatory molecule is the PTP (protein tyrosine phosphatases) SHP-1 (Src homology 2 domain containing tyrosine phosphatase-1). SHP-1 causes inhibition by dephosphorylation of various kinases and their signaling pathways. It has been found that SHP-1 negatively affects JAK2, Erk1/Erk2 MAP kinases, NF-kB, IRF-1, and AP-1, thus inhibiting IFN- γ-inducible macrophage functions (for example, nitric oxide, IL-12 production, and immunoproteasome formation). Other phosphatases (for example, IP3 phosphatase and calcineurin) and surface parasite molecules (for example, LPG) play a major role in the alteration of various second messengers such as Protein Kinase C (PKC), Ca^{2+} (Oliver et al., 2005), inositol lipids, and inositol phosphates, regulating important phagocyte functions (for example, NO and superoxide production).

Effect on electrical functioning of the macrophage plasma membrane

Leishmania parasites alter the electrical functioning of the plasma membrane of the macrophage. This results in hindrance to macrophages activation and signaling of the immune system (Camacho et al., 2008). The lack of activation results in decreased nitric oxide production and decreases outward potassium currents. This may compromise the ability of the macrophage to phagocytose (Berger et al., 1993). The resultant membrane hyperpolarization thus caused decreases NO production (McKinney et al., 1992). Membrane hyperpolarization is associated with decreased TNF-α, altered calcium homeostasis, decreased oxygen radical production, inhibition of cell-cell membrane fusion and prevention of apoptosis (Quintana et al., 2010).

Thus, the macrophages serve as safe-havens for the multiplying parasites. The ability of these parasites to hide within the immune cells has made the design of effective therapies challenging. In addition, species variation and their differing adaptations for intracellular survival results in their unresponsiveness to chemotherapy. Saha et al. (2011), highlighted the participation of various immune cells, microbicidal molecules and altered signaling mechanisms in leishmaniasis, together with the influence of anti-leishmanial drugs which act upon various immune cells like neutrophils, macrophages and lymphocytes.

They inferred that compounds having anti-leishmanial activity could be combined with agents which could modulate the signaling pathways of the host cell for eliciting good therapeutic activity. Strategies have to be devised to target the anti-leishmanial drugs to the macrophages of the liver or the spleen for effective therapy. Thus, the key to successful antilesihmanial chemotherapy is to selectively deliver the active agent either actively or passively to the desired site of action, i.e., the macrophages of the reticuloendothelial system (RES) of the host liver and spleen.

Macrophage targeted drug delivery devices

Suitable strategy for treatment of these diseases is to target the therapeutic agents to the macrophage cells. Incorporation of anti-leishmanial agents in liposomes, nanoparticles, multi-lamellar vesicles, emulsions, microspheres are the new strategies adopted to deliver the drug directly to the parasitophorous vacuole where the parasite resides, thus improving the drug bioavailability and therapeutics. Use of plant derived bioactive molecules which can be entrapped in various carrier systems can also be used as alternative strategies. Colloidal drug carriers were used earlier in microbial diseases involving macrophages. In which a lysomotropic –parasitotropic process for delivery of liposome–encapsulated drugs in the macrophages for targeting *Leishmania* amastigotes (Alving et al., 1988; Agrawal et al., 2000). The process involved uptake of the liposome by phagocytosis and delivery by fusion with the parasitophorous vacuole. Different approaches were adopted with varying successes for chemotherapeutic delivery of the antileishmanial drugs to the cells of the RES by passive or active targeting (Nan et al., 2001).

Liposomes

Remarkable advances in lipid- associated and liposomal nano-drug delivery formulations have been made to reduce the toxicity of the anti-leishmanial drugs in humans. Alving et al. (1978) first demonstrated the utility of liposomes in the treatment of experimental VL in hamsters. Liposome-encapsulated antimonials were found to be 700 times more active than unencapsulated drug, thus confirming the potential of liposomal systems (Date et al., 2007). A drawback of liposomal targeting is their rapid accumulation by the fixed macrophages of the RES. This is advantageous in antileishmanial therapy as the parasite resides in the macrophages of the RES. Due to the potential adverse effects associated with antimonial drugs, liposomal formulations using AmB was developed. Liposomal AmB (L-AmB) was found to be 350 to 750 times more active than meglumine antimonite and 2 to 5 times more active than unencapsulated AmB in experimental leishmaniasis. With the commercialization of L-AmB as AmBisome several studies have been done to demonstrate its safety and efficacy in endemic developing countries. Other clinical preparations like AmB colloidal dispersions (Amphocil) and AmB lipid complex (Abelcet) show minimum toxicity associated with variation in therapeutic index according to geographical locations (Mondol et al., 2010). Out of the three preparations AmBisome demonstrated maximum efficacy and it is the only liposomal product approved for the treatment of leishmaniasis.

Anti-leishmanial effect of plant bioactives; harmine and quercetin in free and liposomal form were evaluated and the liposomal formulations were reported 1.5 to 2 times more effective and considerably less toxic than unencapsulated drugs (Sarkar et al., 2002; Lala et al., 2004). Alterations in liposomal composition and liposomal surface molecules were experimented to improve macrophage uptake. Attempts were made to target antileishmanial drugs encapsulated in mannosylated or fucosylated liposomes to treat experimental leishmaniasis in the hamster model (Sinha et al., 2000). Mannosylated liposomes were more potent in delivering antileishmanial drugs to phagocytic cells. The hepatocytes and the macrophages in the liver have distinct receptors for galactose and mannose. Mannose containing glycosides thus could be directed to macrophages of liver cells. The potential of two different ligands, palmitoyl mannose (Man-Lip) and 4-SO_4GalNAc (Sulf-Lip) to target specifically resident macrophages was investigated after surface modification of AmB loaded liposomes and 4-SO_4GalNAc (Sulf-Lip) was reported for enhanced target specificity (Singodia et al., 2011). Sinha et al. (2000) demonstrated that liposomes loaded with andrographolide reduced the parasitic burden in the spleen, as well as reduced the hepatic and renal toxicity. In addition, mannosylated andrographolide liposomes treated animals showed a normal blood picture and splenic tissue histoarchitecture when compared with those treated with free drug or liposomal andrographolide preparation. Pentamidine isethionate and their analogues were also examined *in vitro* for antileishmanial activity in mannose bearing liposomes (Banerjee et al., 1996). The potential of neoglycoprotein-conjugated liposomes was also established for improving the targeting of hamycin to macrophages infected with *Leishmania* (Date et al., 2007). Neoglycoprotein- conjugated hamycin liposomes eliminated intracellular amastigotes 1.5 to 10 times more effectively than that of unconjugated liposomal and free hamycin. However, though mannose and neoglycoprotein grafting was observed to be effective, the intricacies associated with the grafting process often limit their real field utility.

Some plant glycosides like amarogentin isolated from *Swertia chirata* (Medda et al., 1999) and Bacopasaponin C isolated from *Bacopa monniera* were reported to have antileishmanial properties (Sinha et al., 2002). On incorporation in the liposomes, these molecules serve independent purposes – (1) the end sugar of the hydrophilic sugar chain sticks out of the liposomal surface which acts as ligands for appropriate receptors on the macrophage surface and (2) because of their leishmanicidal properties, they also act as an antileishmanial drug and also (3) the interaction of the drug with non-target tissues is minimized. Basu et al. (2005) used two indigenous glycosides, one having glucose as an end sugar in the hydrophilic sugar chain (eg; acaciaside) and the other having rhamnose as an end sugar with no tissue specificity but having leishmanicidal activity (for example, asiaticoside) were incorporated together into the

liposomes in different molar proportions for testing in experimental leishmaniasis in animal models. Both acaciaside and asiaticoside incorporated liposomes were more efficient in lowering the spleen parasite load compared to asiaticoside. Better uptake of drug by macrophages coupled with improved delivery to liver and spleen was observed when the drug is entrapped in positively charged liposomes than that of negatively charged or neutral liposomes (Miller et al., 1998). Dey et al. (2000) reported that a single dose of cationic liposomes containing phosphatidyl choline (PC) and stearyl amine (SA) could significantly reduce the hepatic parasite burden in experimental leishmaniasis. Although the complete mechanism is not known it was suggested that reversible electrostatic interaction between PC-SA liposomes and parasite plasma membranes cause disruption of the cell membrane and damage cellular organization. Cationic liposomes have also been used to deliver DNA to target cells. Several cationic liposome-encapsulated anti-sense oligonucleotides, complementary to the *Leishmania* universal miniexon sequence or specific sequences like β-tubulin have been tested *in vitro* for reducing parasite burden (Dasgupta et al., 2002). Tubulin synthesis could be inhibited preventing intracellular parasites from multiplying. Cationic liposomes increased the efficacy of anti-sense oligonucleotides nearly three fold compared to that of uncoated oligonucleotides. Macrophage activating peptides (for example, tuftsin) were grafted on the liposome surface. These liposomes not only showed better antileishmanial activity but also acted as immunomodulators by activating the MPS non-specifically against infections (Agrawal and Gupta, 2000). Banerjee et al. (1998) explored the potential of other chemotactic peptide f-Met-Leu-Phe (fMLP)-grafted liposomes for the treatment of VL. Cationic liposomes with *Leishmania donovani* promastigote membrane antigens interacted efficiently with antigen-presenting cells and could trigger CD8+ T cell responses as well (Bhowmik et al., 2010). The surface of macrophages possesses F_c receptors that bind to the F_c portion of antibodies like immunoglobulins (Ig). Hence, when such antibodies are coupled to liposomes or liposomes containing anti-leishmanial drugs, an improvement in macrophage targeting coupled with synergistic anti-leishmanial activity is likely to be achieved (Kole et al., 1999).

Liposomal drug delivery system can be administered through different routes such as, oral, intravenous, subcutaneous, intraperitoneal, intramuscular or inhalation through the bronchial tract (Basu et al., 2005). Orally administered liposomes, pass through stomach digestion, but are lysed by the lipolytic enzymes in the intestine. Intravenously injected liposomes are rapidly cleared from the blood and are absorbed mainly by the phagocytic cells of the RES. Liposomes injected through subcutaneous, transdermal or intramuscular routes may remain in the circulation longer. They may act as a depot for drugs and facilitate the slow release of the entrapped materials from the vesicles. Toxicity studies also showed no apparent drug toxicity in mannose bearing liposomal forms (Basu et al., 2005). Liposomal or sugar grafted liposomal formulations of AmB, 8-aminoquinoline derivatives and antimonial drugs have shown to be more effective against VL and less toxic than free drugs. One major disadvantage of liposomal formulations is its infusion related toxicity due to leakage of the free drug into the systemic circulation (Barratt and Bretagne, 2007). Furthermore, prolonged disposition kinetics of liposomal formulations causes unwanted non-specific accumulation of toxic drugs in the macrophages. To overcome these disadvantages polymeric drug delivery systems were further developed.

Polymeric nanoparticles

Different synthetic and natural polymers with biodegradable and biocompatible characteristics were also explored. N-(2- Hydroxypropyl) methacrylamide (HPMA) copolymer has shown promise in the delivery of an anti-leishmanial 8-aminoquinoline (Nan et al., 2004). Anti-leishmanial activity of poly (HPMA)–amphotericin B conjugates both *in vitro* and *in vivo* was reported (Nicoletti et al., 2009). Polymeric nanoparticles generally described as nanospheres and nanocapsules, have been proposed for use as passive drug delivery to macrophages because of their long circulation time in the body and rapid clearance from the plasma by the mononuclear phagocyte system (MPS) (Kreuter et al., 2005). HPMA-based copolymer drug delivery system (Nan et al., 2004) for leishmaniasis is composed of five components; HPMA-copolymer, targeting moiety (mannose), non-degradable linker for targeting moiety (usually di/tri/oligo amino acids), antileishmanial drug (AmB) and lysosomally degradable linkers like small peptides for active drug. The polymer drug conjugates were synthesized from a polymeric precursor by aminolysis followed by substitution at the terminal amino group of the antileishmanial drug and during synthesis the targeting moiety was introduced. Conjugates were reported nontoxic when tested against mammalian KB cells for cytotoxicity (Nan et al., 2001). Gaspar et al. (1992) was the first to evaluate the potential of primaquine-loaded poly-alkylcyanoacrylate (PACA) nanoparticles against *L. donovani*-infected macrophages.

Primaquine-loaded nanoparticles were reported to be 21 times more effective than the free primaquine in eradicating the *Leishmania* parasite. Paul et al. (1998) linked pentamidine experimentally to methacrylate polymer nanoparticles. *In vitro* studies showed that nanoparticulate pentamidine was 25 times more effective than free drug whereas *in vivo* studies revealed that nanoparticulate pentamidine was much more superior to free drug in reducing parasite burden from liver and also

the side-effects associated with the drug. Basu et al. (2004) prepared pentamidine-loaded poly (D, L-lactide) nanoparticles by the nano-precipitation method. The cytotoxicity on J774 cells were tested using unloaded nanoparticles, pentamidine-loaded nanoparticles, and pentamidine isethionate alone (Paul et al., 1998). The percentage of binding decreased significantly with drug load. A nonlinear increase in drug uptake per unit mass of polymer with the equilibrium pentamidine concentration was found. After 24 h of incubation, pentamidine-loaded nanoparticles presented an IC_{50} value significantly lower than that of free drug (0.39 vs. 6.5 g/ml).

Nanoparticulate systems based on biodegradable poly (ε-caprolactone) have been developed to improve anti-leishmanial action of AmB with concomitant reduction in the toxicity associated with it (Espuelas et al., 2002). Nanoencapsulated AmB was found to be 2 to 3 times more effective than free AmB in reducing parasite burden from Leishmania-infected mice and the side effects associated with AmB. Nanoencapsulation of AmB resulted in abolishment of TNFα release and nitric oxide production by macrophages which are inherent effects shown by free AmB indicating alteration in intracellular trafficking and association of AmB. Nanoencapsulation of anti-leishmanial agents of natural origin has been an innovative strategy to improve the bioavailability of these drugs. Quercetin (Sarkar et al., 2002), Bacopasaponin C (Sinha et al., 2002) and Arjunaglucoside (Tyagi et al., 2005) have been used in free form or encapsulated in various colloidal carriers such as niosomes, microspheres and nanoparticles. These carriers are advantageous because at equivalent therapeutic concentrations, maximum reduction in parasite burden in the spleen was observed when the anti-leishmanial agents were in nanoparticulate form as compared to free drug or drug encapsulated in other colloidal carriers. Oil-in-water emulsions also known as lipid nanospheres (LN) or fat emulsions were developed for delivering piperine for the treatment of VL. Piperine was formulated in a lipid nanosphere (with stearylamine in one formulation and PEG in the other formulation) and was injected intravenously to BALB/c mice infected with *L. donovani* or 60 days. It was observed that lipid nanospheres encapsulated with stearylamine showed maximum reduction of parasitic load in liver and spleen when compared to the other two formulations (Veerareddy et al., 2004). Pharmacokinetics of piperine in lipid nanospheres showed a biexponential decline with significantly high AUC, a lower rate of clearance and a smaller volume of distribution than piperine.

Nanoencapsulation of antileishmanial plant drug andrographolide in Poly(d,l-lactide-co-glycolide) PLGA was studied in our laboratory in order to improve the antilieshmanial efficacy and bioavailability of poorly soluble bioactive (Roy et al., 2010). Nanoparticles in a size range of 150 to 200 nm demonstrated an increased localization in macrophages predominantly infested with leishmanial parasite (Vasir et al., 2005). Besides, the phagolysosomal acidic pH accelerated degradation of PLGA (Mundargi et al., 2008) promoting specific release of drug in the vicinity of the amastigotes. PLGA nanoparticles (NPs) for the antileishmanial saponin β-aescin were tested *in vitro* for anti-leishmanial activity. The aescin-loaded NPs were more effective than that of free β-aescin in terms of therapeutic efficacy (Van de Ven et al., 2011).

Niosomes

Niosomes are mixtures of non-ionic surfactants and cholesterol which behave like liposomes *in vivo*. Compared to phospholipids used in liposomes, the synthetic non-ionic surfactants used in the preparation of niosomes are chemically stable, precise in chemical composition and cheaper in cost. When tested *in vivo*, the retention capacity of niosomes was found to be higher due to the absence of lipid molecules and their smaller size. Thus the therapeutic efficacy of certain antileishmanial compounds was found to be better. The niosomes, being cheaper, less toxic, biodegradable and non-immunogenic, were considered suitable as drug carriers (Basu et al., 2004). Nieto et al. (2003) studied the effect of niosomal formulation of sodium stibogluconate (NIV-SSG) formulation in dogs and observed that the antileishmanial activity of the drug was appreciably enhanced in the NIV-SSG form and even more in niosomes covered with dextran. Other encapsulations of sodium stibogluconate in niosomes were tested in BALB/C mice and it was observed that the antileishmanial efficacies were NI-SSG-dextran > NI-SSG > free SSG in experimental visceral leishmaniasis (*L. donovani*) (Mullen et al., 1998).

Other drug delivery devices

Various other drug delivery devices consisting of nanodisks, loaded with AmB were tested on experimental cutaneous leishmaniasis in BALB/c mice upon intraperitoneal administration. Veerareddy et al. (2009) developed uncoated and mannose-coated lipid nanospheres of AmB. These formulations were administered in *L. donovani* infected BALB/c mice at a dose of 5 mg/kg body weight. The same dose of uncoated AmB lipid nanospheres and Fungizomes was also administered in separate mice as control groups. The AmB loaded nanospheres improved the capability of the drug to interact with ergosterol. Nanospheres did not show any improvement of the AmB activity against the resistant strain when characterized in the absence of ergosterol **(**Espuelas et al., 2002).

Conclusion

Leishmaniasis is still one of the most neglected diseases

and its treatment remains a challenge because of the prevalence of drug-resistance, high drug-dosage, adverse side-effects, and lack of affordable new anti-leishmanial drugs. Significant attempts have been made to develop low-cost drugs with minimum side-effects but still the morbidity and mortality from visceral leishmaniasis is fast increasing. Pentavalent antimonial drugs have been used for years in treatment of leishmaniasis, but the increasing drug resistance and the side-effects coupled with increasing risk of HIV co-infections have led to a need for advanced therapeutics and early diagnostic techniques. Plant derived antileishmanial compounds have attracted global attention due to their alternative mechanism of action, inherent safety, easy availability and cost-effective nature. Most of these however suffer from poor bioavailability, low solubility and require a high dose for effective therapy. Our investigation with antileismanial phytochemical andrographolide suffering from bioavailability problems showed improved biological efficacy when incorporated in PLGA nanoparticles.

For effective control of leishmaniasis, the strategy construed is to target bioactives to the phagolysosomes of the macrophage where the amastigotes localize, by exploration of different delivery technologies. With the emergence of tailor made targeted delivery devices (DDS) like nanoparticles specific transport of the drug to the target cell can be achieved without affecting the host cell thus minimizing the toxic effects to normal cells. The use of colloidal drug delivery devices such as liposomes, niosomes, nanoparticles, nanospheres to deliver anti-leishmanial agents such as amphotericin, pentamidine, primaquine, 8-aminoquinoline to the target site is being studied extensively. These approaches have established enhanced efficacy and tolerability of antileishmanial drugs with narrow therapeutic indices like amphotericin B. Recent advances in the field of solid lipid nanoparticles and nanostructured carriers are proving to be promising because of their stability and ease of commercialization. DDS reduce drug intake with significant reduction of drug associated toxicity. DDS can increase the bioavailability, solubility, and retention time of many potent antileishmanials that are difficult to deliver orally. Introduction of innovative nano-scale delivery of therapeutics at predetermined target sites are dominating the drug delivery advancements worldwide and may evolve as an effective strategy for leishmaniasis treatment. But further investigations in this line are still beckoned to arrive at an affordable and effective therapy for this neglected tropical disease.

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