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Drug delivery strategies for therapy of visceral leishmaniasis

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Importance of the field: Visceral leishmaniasis (VL) is the most overwhelming type of leishmaniasis associated with the poverty of developing countries and usually mortal if untreated. Most of the conventionally used dosage forms offer us the shortcomings of toxic side effects and emergence of drug resistance. Several efforts have been made to overcome the barriers involved in the treatment of VL. Colloidal carriers extensively represent the drug delivery systems (DDSs) for intracellular localization of antileishmanial compounds in macrophage-rich organs such as liver, spleen and bone marrow. These DDSs offer superior therapeutic efficacy over the conventional treatment in terms of site-specific drug delivery with reduced side effects. However, after 35 years of research in the field, AmBisome® (Amphotericin B liposome for injection, Astellas Pharma US, Inc.) is the only DDS used against the VL.
Areas covered in this review: A literature search was performed (for drugs and DDSs against VL) on PubMed and through Google.
What the reader will gain: This review aims to describe the pathophysiology of VL and its current conventional treatment with special reference to DDSs designed against VL.
Take home message: On reviewing the conventional drugs and DDSs developed against VL, it is concluded that advances in the field of targeted drug delivery can result in more efficient strategies for the therapy of VL.

Keywords: active targeting, colloidal carriers, passive targeting, visceral leishmaniasis

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1. Introduction

Leishmaniasis is a zoonotic infection caused by parasitic protozoans and transmitted by means of sandfly bite to vertebrate hosts including man. The disease is named after British pathologist William Boog Leishman, who first described it in 1903 in London. *Leishmania* are the obligate intracellular parasites responsible for leishmaniasis. Visceral leishmaniasis (VL) is the most severe of all the forms of leishmaniasis and usually lethal if left untreated. The affected sites are the macrophages of liver, spleen and bone marrow. Visceral leishmaniasis also increases the risk of other secondary infections. The pathogens causing VL are *Leishmania donovani* (Asia and Africa), *Leishmania infantum* (Southern Europe) and *Leishmania chagasi* (South America). However, *L. donovani* is the major pathogen and most (~ 90%) VL cases occur in just six countries: India, Bangladesh, Nepal, Brazil, Ethiopia and Sudan. India alone contributes 40 – 50% of these, with 90% of cases occurring in Bihar only [1,2]. In the recent epidemics of Sudan and India, VL resulted in > 100,000 deaths. In India, where VL is also known as Kala azar, high incidences of VL occur in the states of Bihar, Assam, West Bengal and Eastern Uttar Pradesh [3]. According to the World Health Organization (WHO), there are 2 million new cases of leishmaniasis every year. With the introduction of AIDS, the nature of leishmaniasis has also been changed. Leishmaniasis, as an HIV-associated infection, is causing high morbidity and mortality worldwide [4,5].

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Article highlights.

- VL is the most severe of all the forms of leishmaniasis, characterized by high fever, hepatosplenomegaly, hypergammaglobulinemia, thrombocytopenia, diarrhea, and so on.
- Various receptors involved in the interaction of *Leishmania* parasite with the host macrophages are CR1, CR3, CR4, Fc receptors, fibronectin receptors, mannose receptors and the advanced glycosylation end product receptors.
- The amastigote form of parasite is responsible for infecting the entire RES of mammalian host.
- Conventional chemotherapy of VL involves the treatment with pentavalent antimonials, pentamidine, AmB, paromomycin, miltefosine, sitamaquine, and so on.
- Various DDSs (liposomes, emulsomes, niosomes, polymeric particles and conjugates, etc.) are developed and used for passive targeting of drugs to the macrophages for the treatment of VL.
- Ligands that bind to receptors that are expressed over macrophages can be attached to DDSs in an attempt to increase further macrophage accumulation. Ligands may also increase macrophage uptake of carrier-encapsulated drugs by receptor-mediated endocytosis of intact carriers.
- The commonly used natural plant products for antileishmanial activity are alkaloids, terpenes, phenolics, chalcones, and so on. These plant products can also be used in the form of carriers for the treatment of VL.

This box summarises key points contained in the article.

Visceral leishmaniasis has sinister inception with pyrexia, which is continuous or remittent and becomes intermittent at a later stage. It normally doubles in 24 h. Children presenting later in the course of the disease may present with edema caused by hypoalbuminemia, hemorrhage caused by thrombocytopenia, or growth failure caused by features of chronic infection. Visceral leishmaniasis is typically characterized by high fever, hepatosplenomegaly, hypergammaglobulinemia, jaundice, lymphadenopathy, anemia, leukopenia, thrombocytopenia and diarrhea. As the disease progresses, the skin of the hands, feet, abdomen and face may become darkened, giving VL the name kala azar or black fever [6,7].

2. Interaction of *Leishmania* parasite with the host macrophages

Leishmania parasite exists in two forms: flagellated, motile, elongated promastigotes and round, non-motile amastigotes. The promastigotes reside in the mid-gut of the sandfly and amastigotes in the mammalian host [8]. Promastigotes often coming in contact with host macrophages are phagocytosized by means of a receptor-mediated mechanism and are taken up into the phagosome, which fuses with lysosome to form the phagolysosome. Inside the macrophages, the promastigotes are converted into the amastigote form. This change occurs as a result of momentous biochemical and metabolic changes in the promastigotes. Amastigotes are further released from macrophages and can reinvade other macrophages [9].

2.1 Phagocytosis of promastigotes by macrophages

Phagocytosis is the process of engulfment of any foreign material by the macrophages. However, *Leishmania* parasite can resist the microbicidal activity of macrophages. Phagocytosis of promastigotes is comprised of two allied proceedings: i) attachment via low affinity, rapid kinetics interactions; and (ii) internalization following a high-affinity interaction. The uptake of promastigote by macrophages may occur by any of two mechanisms – zipper or coiling. In the zipper mechanism, the attachment of the parasite to a phagocyte receptor triggers the activation of more receptors from the contiguous membrane, forming a pseudopod, which advances along the parasite like a zipper, hence forcing the promastigotes into the phagosome. In the coiling mechanism various pseudopods are arranged asymmetrically [9,10], delivering the parasite to a cytoplasmic compartment where its survival becomes uncertain [11].

2.2 Promastigote macrophage ligand receptor system

Complement receptors CR1 and CR3 play an important role in phagocytosis of promastigotes. The parasite may interact with complement receptors in three ways: i) activating the complement component C3 and binding through the C3bi fragment of complement to CR3 in the presence of serum; ii) a serum-independent approach of binding of the surface protease gp63 to CR3; and iii) binding of parasite lipophosphoglycan to the lectin-like site on CR3 and to CR1 [12]. The survival of parasite improves by the involvement of complement receptors as it prevents the respiratory burst [13,14]. Various other receptors such as CR4, fibronectin receptor, mannose receptor and the advanced glycosylation end product receptor have also been reported to be engaged in the process of phagocytosis [15].

Major ligands present on promastigotes for phagocytosis are gp63 and the phosphoglycans [15]. A zinc metalloprotease, gp63, presents on the surface of promastigotes but not on amastigotes. The phosphoglycans include lipophosphoglycans (LPG) and proteophosphoglycans (PPG). LPG, a glycolipid present in very dense population on the promastigotes, contains repeating units of Gal ($\beta 1 - 4$) Man ($\alpha 1$-) PO4 and may or may not contain supplementary glycan side chains. PPG also contain a similar arrangement of glycan units as in LPG [16,17], as well as leucine at their N terminus [18].

2.3 Amastigote interaction with macrophages

In *L. donovani*, LPG is absent in amastigote form [19], thus PPG is involved in the phagocytosis of parasite by the macrophages. Macrophages possess different receptors for specific ligands on amastigotes such as CR3 receptors, Fc receptors and mannose receptors [20]. The dendritic cells play an important role in the transport of amastigotes from the

infected site to the draining lymph nodes, where parasite is presented to T cells [21]. After the amastigotes' entry into the macrophages, they multiply continuously until the macrophage bursts and the parasite is released in the surroundings. However, the mechanism of release of amastigotes from the macrophages is still unclear.

2.4 *Leishmania*-containing parasitophorous vacuoles

As the parasite comes into contact with macrophages, it is picked up into a membrane-bound phagosome that is contiguous with the plasma membrane of the macrophages. The phagosome then fuses with lysosome, to form phagolysosome or parasitophorous vacuole (PV) [22]. Parasitophorous vacuole may also be defined as a microbicidal peptide and hydrolytic enzyme-rich acidic section [23]. However, infection of *Leishmania* inhibits the production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in the PV [24]; the pH of vacuole is maintained [23,25].

3. Pathophysiology

The female sandfly infects itself with the *Leishmania* parasites by sucking blood from the mammalian host. During a period of 4 – 25 days, the parasite continues to multiply by binary fission inside the mid-gut of the sandfly and undergoes major transformation into the promastigote form. The promastigotes tend to migrate to the pharynx and buccal cavity of the sandfly. A heavy pharyngeal infection is observed between the sixth and ninth day of an infected blood meal. Leishmaniasis spreads by the bite during this period. After the bite, some of the promastigotes entering the circulation are destroyed whereas others are taken up by the macrophages. Here they change into the amastigote form. The amastigotes multiply continuously by binary fission inside the macrophages, until the macrophages rupture, liberating the amastigotes into the circulation. The free amastigotes repeat the cycle by invading other macrophage cells and infect the entire reticuloendothelial system (RES). Some of the free amastigotes are drawn by the sandfly during its blood meal, thus completing the cycle (**Figure 1**) [6,26,27].

4. Conventional chemotherapy

Drugs are the major treatment available against VL that can erode this fatal infection from society. Conventionally available chemotherapy for VL is facing challenges owing to the development of resistance in the parasites to the most common antileishmanial drugs used because of incomplete treatment schedules or reduced drug doses taken by the patients owing to fear of toxicity. Most of the conventional dosage forms, after administration, deliver the drug into the body, which ultimately reaches the site of action by distribution and passive diffusion, consequently giving rise to side effects [28]. Moreover, high drug clearance from the body also limits the use of conventional drugs. Also, the current treatments require a prolonged course and treatment is often conducted in a hospital setting.

4.1 Parenteral chemotherapy

4.1.1 Pentavalent antimonials

Throughout the world pentavalent antimony compounds (Sb^v) are used as first-line therapy for the treatment of VL [29]. Two major antimonials used at present are sodium stibogluconate (SSG) and meglumine antimoniate. SSG contains 100 mg/ml antimony and is used primarily in English-speaking countries. Its closely related compound meglumine antimoniate contains 85 mg/ml antimony and is used primarily in French-speaking countries. The drugs are given intravenously or intramuscularly and are equal in efficacy at equivalent doses. The recommended regimen consists of once daily injection of full drug dose (20 mg/kg) for 30 days. Although active elsewhere in India, antimonials are no longer useful in the northeastern state of Bihar, where as many as 65% of previously untreated patients fail to respond to or promptly relapse after therapy with antimonial compounds [30]. Disadvantages of antimonials include the parenteral mode of administration, the long duration of therapy and adverse reactions. Systemic toxicity is normally related to total dose administered. Secondary effects are fatigue, bodyache, electrocardiographic abnormalities, raised aminotransferase levels and chemical pancreatitis. However, sudden death resulting from arrhythmia has been reported in patients receiving high doses for a prolonged period [31,32].

Previously a dose of 10 mg/kg for 6 – 10 days was effective but increasing resistance has led to a consecutive rise in the dose size to > 10 times [33]. However, the response varies from region to region. A study by Sundar *et al.* [30] in different endemic regions of India has shown that Sb^v treatment in Bihar (India) was unresponsive in ~ 60% of previously untreated patients, whereas the drug had a response in Uttar Pradesh. The accurate mechanism of action of the drug is still unknown. Researchers believe that Sb^v inhibits glycolysis, fatty acid beta-oxidation and ADP phosphorylation in the parasite [34,35]. A recent report indicates that all pentavalent antimonials (Sb V) require biological reduction to the trivalent (Sb III) form for antileishmanial activity [36,37]. Wyllie *et al.* [38] reported the dual action of antimonial drugs on thiol redox metabolism, which rendered the parasite more susceptible to oxidative stress.

4.1.2 Pentamidine isethionate

Pentamidine, an aromatic diamidine, has been very useful in Sb^v-resistant VL [39]. Bray *et al.* [40] reported that the drug inhibits arginine transport and influences the polyamine biosynthesis, which is responsible for its antileishmanial activity. A dose of 4 mg/kg given 3 times a week for 3 – 4 weeks resulted in a 98.8% cure of the disease, but the response to the drug declined to ~ 75.2% after a few years [41]. Further use of this drug leads to adverse effects, such as tachycardia, hypotension and hypoglycemia [41,42], which become more severe as it causes irreversible insulin-dependent diabetes

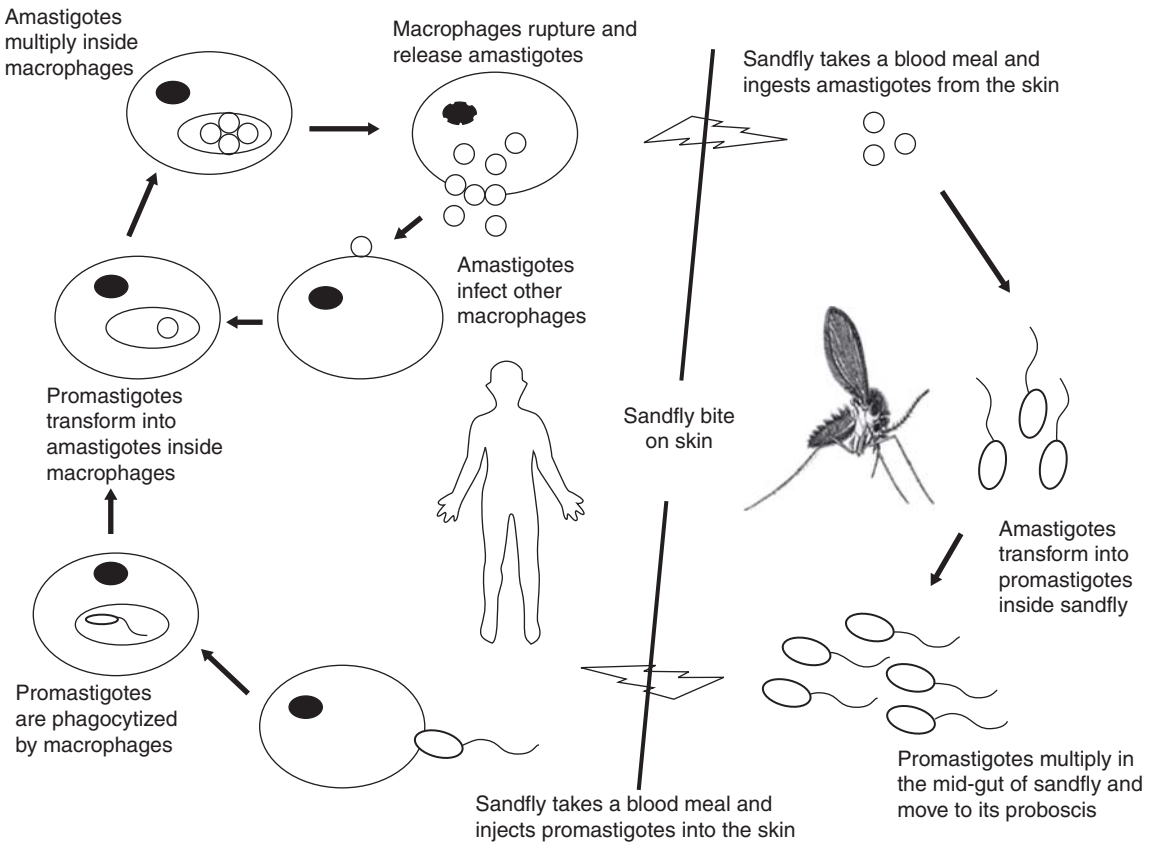


Figure 1. Life cycle of *Leishmania* parasite.

mellitus and possibly death. Moreover, the high cost led to total ban of the drug in India.

4.1.3 Amphotericin B and its lipid formulations

Amphotericin B (AmB), a polyene macrolide antibiotic, is a gold standard drug for the treatment of VL. AmB was discovered in 1956 and obtained from *Streptomyces nodosus*, actinomycetes obtained from the soil of Orinoco river in Venezuela. AmB is a hydrophobic molecule, insoluble in water and poorly soluble in most organic solvents. It is an amphipathic molecule, having a hydrophilic polyhydroxyl head and a hydrophobic polyene tail. Fungizone, an AmB-deoxycholate (AmB-Doc) complex, was the first commercial preparation of AmB. AmB is administered intravenously as it is poorly absorbed by the gastrointestinal tract. The preferred dose of drug is 1 mg/(kg day) for 20 days [43]. The activity of AmB depends principally on its binding to sterol moiety, primarily ergosterol, present in the membrane of the *Leishmania* parasite but not in mammalian cell membranes. By virtue of its interaction with ergosterol, it forms pores or channels that increase the membrane permeability and allow leakage of a variety of intracellular constituents and thus kills the parasite. AmB is not frequently used because of its side effects such as fever, chills, cardiac arrest, hypokalaemia and nephrotoxicity [44,45]. AmB administered at a dose of 0.75 – 1 mg/kg for 15 – 20 infusions either daily or on alternate days resulted in 97% success in the treatment of VL [46]. Mishra *et al.* [47] reported 100% cure rate with a daily dose of 0.5 mg/(kg day) in patients who had not received Sb^v for 14 days. The same results were obtained after administration of 1 mg/kg every other day, for a total of 20 injections in children [48]; but prolonged course and high cost of treatment, associated with toxic side effects such as high fever with rigour and chills, myocarditis, severe hypokalaemia, renal dysfunction and even death, led to a decline in the use of this drug.

To reduce the side effects of the drug, lipid formulations of AmB were developed. The new formulations of AmB include liposomal AmB (Ambisome, L-AmB) [49,50], AmB colloidal dispersion (Amphocil, Amphotec, ABCD) [51] and AmB lipid complex (Abelcet, ABLC) [52]. Ambisome, a liposomal preparation, consists of small unilamellar vesicles (60 – 70 nm in size) of phospholipids such as phosphatidylcholine or distearoylphosphatidylglycerol that are stabilized by cholesterol. Amphocil or Amphotec, a disc-like structure (~ 15 nm in diameter), is formed by complexation of cholestryl sulfate with AmB in the ratio 1:1. Abelcet is a ribbon-like molecular structure obtained by combination of dimyristoylphosphatidyl choline and dimyristoylphosphatidylglycerol (molar ratio of 7:3) with AmB [43,53].

All the formulations have been tested successfully throughout the world, even with HIV co-infected patients [46]. Ambisome, the first to be evaluated and licensed in many European countries and the US for treatment of VL, is recommended at a dose of 3 mg/kg on days 1 – 5, 14 and 21, for a total dose of 21 mg/kg [54]. The cure rate was found to be 90 – 100% in 10 days of therapy with a total dose of 18 – 24 mg/kg in Europe and South America and with 14 – 18 mg/kg in Kenya [55]. In India 100 and 89% cure rates were obtained with doses of 6 and 3.75 mg/kg, respectively [56,57]. Acute side effects were observed with a single dose of 7.5 mg/kg of Ambisome; however, the cure rate was found to be 90% [58]. Amphocil at a dose of 15 mg/kg given for 5 – 10 days cured 90 – 100% of patients [59,60]. Similarly, Amphocil at a dose of 2 mg/kg given 5 and 7 times showed 90 and 100% cure rates [61,62].

Out of these three lipid formulations, Ambisome is the best tolerated, but the limiting factor is the high cost. AmBisome is available in developing countries for VL at a WHO-negotiated price of $20 per 50 mg vial, as compared with conventional AmB treatment of $1.9 per 50 mg vial [63]. This high cost is unaffordable by the patients of developing countries, hence limiting the use of these formulations, but Médecins Sans Frontières can now afford it in its Indian treatment programs for VL.

4.1.4 Paromomycin

Paromomycin, an aminocyclitol-aminoglycoside antibiotic is used for the treatment of VL either alone or in combination with Sb^v. Paromomycin acts on *L. donovani* by interfering with ribosomes, thus inhibiting protein synthesis [64]. The drug may also act by inducing respiratory dysfunction in promastigote form of the parasite [65]. Oral as well as locally applied paromomycin has been used in the treatment of cutaneous leishmaniasis, whereas for VL paromomycin needs to be injected parenterally. A dosage regimen of 14 – 16 mg/(kg day) given up to 3 weeks showed 79% cure of VL [66]. In a study by Sundar *et al.*, paromomycin (final cure rate, 94.6% at a dose of 11 mg/kg (body weight) injected intramuscularly daily to 502 patients for 21 days) was shown to be non-inferior to AmB (final cure rate, 98.8% at a dose of 1 mg/kg injected intravenously to 165 patients every other day for 30 days) [67]. Some other drugs under investigation as future drugs for VL are buparvaquone, tafenoquine, sertraline, tamoxifen, niacin, azithromycin, and so on [63].

4.1.5 Combination therapy

Combination therapy has commonly been used for the treatment of various infectious diseases such as malaria and tuberculosis, and this motivates the therapy to be the same approach for VL. Combination therapy of different drugs with different mechanisms of action has a broader clinical efficacy. Moreover, combinations also reduce toxicity as lower doses of the individual drugs are effective. Combining SSG with paromomycin may be an efficient alternative to single agent therapy. Several studies show the superior efficacy of combination therapy as compared with SSG alone [68-70]. Paromomycin in combination with SSG when given for 20 days cured 82% patients in India and reduced the total time of treatment to about half as compared with SSG alone, when tested in Sudan [44,71].

Melaku *et al.* [72] studied the combination therapy of paromomycin and SSG for the treatment of VL in southern Sudan. They compared the 17-day regimen of SSG combined with paromomycin and 30-day SSG monotherapy and found that the initial cure rate among patients treated with combination was 97.0% compared with 92.4% among patients treated with SSG monotherapy.

In a pilot study in Bihar, India, a 20-day drug regimen of paromomycin (12 mg/(kg day)) in combination with SSG (20 mg/(kg day)) proved efficacious and well tolerated in patients with VL. Out of 22 evaluable patients, 18 achieved an ultimate cure. The remaining four patients, although not cleared of parasites, had their parasite grade reduced and also improved clinically [73].

Thakur *et al.* [74] designed a trial in Bihar, India, to assess the safety and efficacy of paromomycin (12 or 18 mg/kg daily) plus SSG (20 mg/kg daily) for 21 days compared with SSG alone for 30 days. At the end of treatment, 49 of 52 patients receiving paromomycin (12 mg/kg) + SSG, 46 of 48 receiving paromomycin (18 mg/kg) + SSG, and 27 of 49 patients receiving SSG alone were cured. During follow-up there was 1 relapse in each of the treatment groups, giving final cure rates of 48 of 52 (92.3%) for paromomycin (12 mg/kg) + SSG, 45 of 48 (93.8%) for paromomycin (18 mg/kg) + SSG, and 26 of 49 (53.1%) for SSG. Paromomycin plus SSG for 21 days at either 12 or 18 mg/kg daily was significantly more effective than SSG alone for 30 days. Paromomycin 12 or 18 mg/kg daily plus a standard dose of SSG for 21 days were found to be statistically more effective than SSG in producing a final cure for patients with VL.

Recently, in a study in India, a combination of Ambisome (3.75 or 5 mg/kg) and miltefosine was used and cure rates > 90% were found in the VL-infected patients, irrespective of the duration of days of miltefosine treatment (7, 10 or 14 days) [63,75].

4.2 Oral chemotherapy

4.2.1 Miltefosine

Miltefosine, an alkylphospholipid derivative, is an orally effective antileishmanial agent and is found to be cytotoxic to both promastigote and amastigote forms of *Leishmania* [76-78]. Miltefosine was registered in March 2002 in India for treatment of VL [33]. The drug showed excellent results in a Phase II multi-centric clinical trial in India in which 120 adult patients were treated with doses of 50, 100 or 150 mg/day. Out of 120 patients treated, 114 patients were completely cured and 6 patients relapsed within 6 months [79]. In another study, at a daily dose of 2.5 mg/kg for 28 days, 94% of patients were cured [80,81]. However, miltefosine is the

only oral chemotherapy available against VL; its use is still limited as the drug is teratogenic and also causes severe renal toxicity. Moreover, its prolonged half-life is also an issue of concern for the emergence of drug resistance [82]. Sundar *et al.* [83] performed a clinical trial in India comparing miltefosine with AmB. The study was a randomized, open-label comparison, in which 299 patients of 12 years age or older received orally administered miltefosine (~ 2.5 mg/kg (body weight) daily for 28 days) and 99 patients received intravenously administered AmB (1 mg/kg every other day for a total of 15 injections). The results showed that, at the end of study when splenic aspirates were obtained from 293 patients in the miltefosine group and 98 patients in the AmB group, no parasites were identified, for an initial cure rate of 100%. By 6 months after the completion of treatment, 282 of the 299 patients in the miltefosine group and 96 of the 99 patients in the AmB group were cured.

4.2.2 *Sitamaquine*

Sitamaquine, an orally active 8-aminoquinoline analogue (WR 6026), was developed by Walter Reed Army Institute (US) in collaboration with GlaxoSmithKline. The compound, developed earlier for malaria, later showed hopeful results against VL when studied on animal models [84,85]; but when human trials were performed in Kenya and Brazil, the cure rate obtained was only 50 and 67%, respectively. In India sitamaquine at a daily dose of 1.75 – 2 mg/kg for 28 days showed excellent leishmanicidal properties [86]. The drug at higher doses is reported to be nephrotoxic [87]. The details of various conventionally used drugs against VL are summarized in **Table 1**.

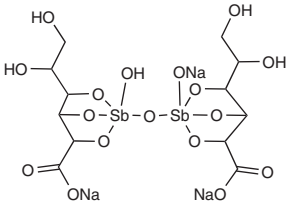
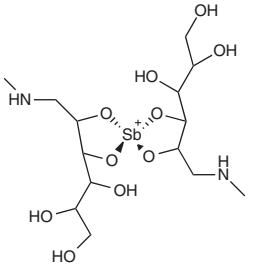
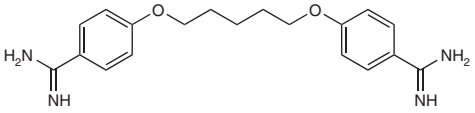
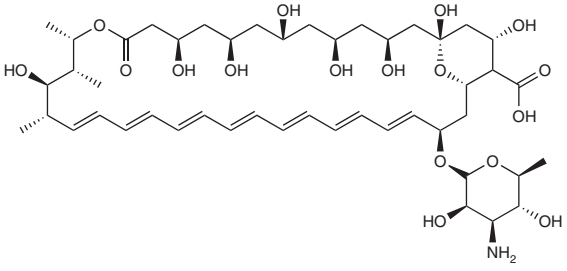
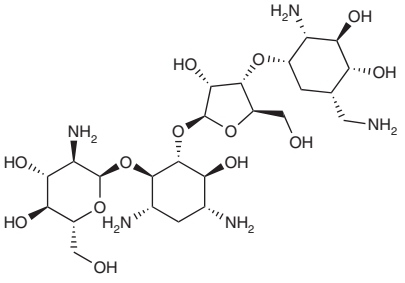
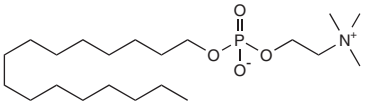
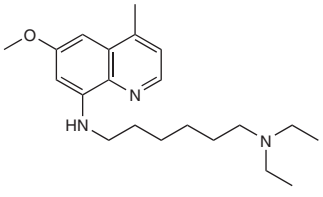
5. Carrier-based passive targeting to macrophages for the treatment of visceral leishmaniasis

When drugs are administered in free form in the body, only a small fraction can reach the macrophages, which in turn may lead to toxic side effects. This serious problem necessitates the development of various strategies for selective and preferential delivery of drugs to the macrophages. Control over spatial and temporal distribution of drug molecules after systemic or localized administration represents the main challenge in drug delivery. Whereas pharmacokinetics can be determined to some extent by the rate of drug administration into the body, the spatial drug profile in various organs or its biodistribution is much more difficult to control. Typically for systemic applications, pharmacokinetics can be influenced by mechanical devices and biodistribution mostly by drug delivery systems (DDSs). Chemotherapy and especially systemic administration of drugs is plagued by insufficient drug delivery to the desired site and toxic side effects, because there is practically no control over biodistribution of systemically administered drugs. Dosing and the use of mechanical devices, such as minipumps, microreserviors with controlled leakage, skin patches, suppositories, and so on, can change mostly pharmacokinetics, that is, temporal concentration profiles of the drug in the body, but in general does not influence biodistribution to any important extent. For example, infusion pumps or slow infusion can avoid peak levels of a drug administered as a bolus and provide sustained drug levels, but lack control of the disposition of the medicament. By contrast, colloidal drug carriers can substantially influence not only pharmacokinetics but also biodistribution of the drug [88]. This category of targetable devices includes drug-bearing bilayer vesicular systems (liposomes, niosomes, pharmacosomes, virosomes) as well as particulate carriers in the micrometer or sub-micrometer size ranges (microparticles, nanoparticles, magnetic microspheres, albumin microspheres, nanocapsules, albumin nanospheres, solid lipid nanoparticles). The ability of some colloids to be taken up by the macrophages of RES especially in liver and spleen has made them ideal vectors for passive targeting of drugs to these compartments (**Figure 2**). The passive targetability attributed to these microparticulate drug carriers is due to the recognition of these exogenous particles either in intact or in the opsonized form by the phagocytic cells of the RES, and this sensing behavior is exploited to target macrophage-associated diseased cell lines [89]. Colloidal carrier systems have been largely studied for the treatment of VL because both colloidal carriers and *Leishmania* parasite are taken up by the macrophages, creating an ideal situation for a high degree of drug parasite interaction, hence providing better therapeutic effects.

5.1 Liposomes

In 1965, Bangham *et al.* [90] reported that the small closed vesicular structures consisting of lipid bilayers could be formed when phospholipids are hydrated by the addition of water. These structures were first named as 'smectic mesophases' by Bangham and later called 'liposomes' by Gerald Weissman [91,92]. Liposomes are microscopic vesicular bilayered structures, which are biocompatible, biodegradable and non-immunogenic. Generally, liposomes can be classified as multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). Naturally occurring phospholipids are the main components of the bilayers, which make them non-toxic and biodegradable. Liposomes have the ability to entrap drugs both in an aqueous and in a lipid phase, making themselves an attractive delivery system for both hydrophilic and hydrophobic drugs. Drugs incorporated into liposomes remain protected from degradation by the external environment of the body. High entrapment efficiency and reduced drug toxicity further make them an ideal carrier for drug delivery. They can control the delivery of drugs by targeting the drugs to the site of action or by site avoidance drug delivery or by prolonged circulation of drugs [93]. Drugs incorporated in liposomes have also been shown to distribute mainly to reticuloendothelial tissues including liver, spleen and lungs [94].

Table 1. Various conventional drugs used against visceral leishmaniasis, their structure, dose and side effects.

Drug	Structure	Dose, route of administration	Side effects	Ref.
SSG		20 mg/(kg day) in 2 divided doses for 30 days, i.m. or i.v.	Cardiotoxicity	[33,37,43,198]
Meglumine antimoniate				
Pentamidine		2 – 4 mg/kg for 15 days, i.m. or i.v.	Unacceptable toxicity and insulin-dependent diabetes mellitus and maybe death	[33,43,199]
AmB		1 – 3 mg/kg for 20 days, i.v.	Nephrotoxicity, cardiac arrhythmia and hypokalemia	[33,43]
Paromomycin		15 mg/(kg day) for 21 days, i.m.	Renal and eighth cranial nerve toxicity	[44,200]
Miltefosine		2.5 mg/(kg day) for 28 days, oral	Teratogenicity, hepatotoxicity, vomiting and diarrhea	[33,43]
Sitamaquine		1.75 – 2.5 mg/kg for 28 days, i.m.	Renal toxicity	[43,185]

i.m.: Intramuscular; i.v.: Intravenous.

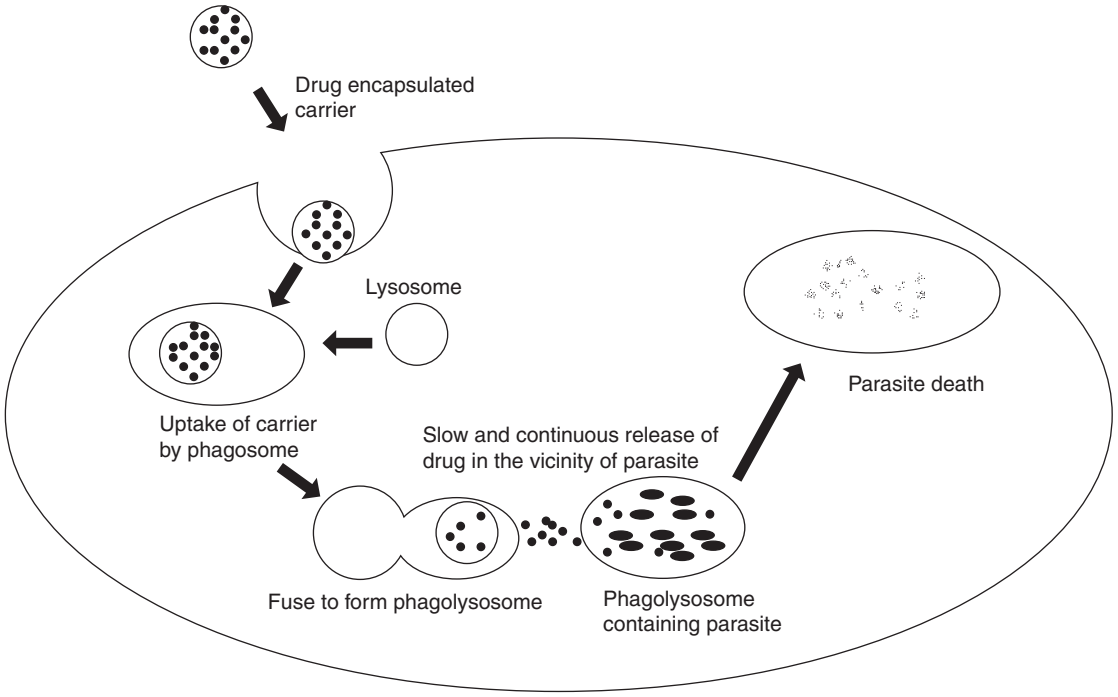


Figure 2. Schematic representation of carrier-based passive targeting to macrophages for the treatment of visceral leishmaniasis.

5.1.1 *Liposomes as lysosomotropic drug carriers*

Liposomes are also defined as 'lysosomotropic' drug-carrying particles [95]. In leishmaniasis the lysosomes themselves serve as active carriers for delivering the liposomes to the parasites. Therefore, this process might be viewed as a 'lysosomotropic-parasitotropic' mechanism. Release of drug in the vicinity of the parasite results in disintegration of the parasite. As a result of the close and continuing interactions and fusions between phagosome and lysosomes, liposomes are brought in a steady flow into the immediate vicinity of the parasite, and even within the parasitophorous vacuole (phagolysosome) enclosing the parasites. In the most intimate situation the liposomes apparently are even injested by the parasites and probably enter the lysosomes within the parasites themselves. By analogy with previously coined terminology, this last step (**Figure 3**) is referred to as a 'lysosomotropic-lysosomotropic' mechanism, and the overall process is referred as a 'lysosomotropic-parasitotropic' mechanism of drug delivery [96].

5.1.2 *Role of liposomes in targeting visceral leishmaniasis*

Liposomes have been used extensively as drug carriers for the selective and targeted delivery of various antileishmanial drugs. At first this strategy was utilized by Black *et al.* [97] and New *et al.* [98] to deliver selectively the pentavalent antimony to the liver and spleen macrophages infected with VL. Then, Alving *et al.* [99] also demonstrated that liposome-encapsulated antimonial drugs (meglumine antimoniate and SSG) were 700 times more effective as an antileishmanial agent than the corresponding antimonial drugs alone, when tested in *L. donovani*-infected golden hamsters.

Soon after, New *et al.* [100] investigated several antiparasitic agents for their antileishmanial activity in a *Leishmania* model. The drugs were administered intravenously, both alone and in the liposomal form. The compounds, such as griseofulvin, 5-fluorocytosine and AmB, were tested together with the known antileishmanial drug pentamidine. The results displayed superior therapeutic activity in liposomal form. Liposomes of AmB composed of hydrogenated lecithins, and sterols in the membrane, were found to be least toxic with utmost antileishmanial activity.

In 1984, Chapman *et al.* [101] determined the antileishmanial efficacy of liposome-encapsulated meglumine antimoniate in *L. donovani*-infected dogs. A single injection (0.61 mg of Sb/kg (body weight)) of this formulation resulted in 89% suppression of splenic parasites, whereas 4 consecutive daily injections (1.94 mg of Sb/(kg day)) of formulation resulted in 95.8% suppression of splenic parasites.

Further, the antileishmanial activity of liposomal AmB was tested in hamsters and monkeys by Berman *et al.* [102] and the results demonstrated that > 99% of *L. donovani* parasites were eliminated from the liver and spleen of infected hamsters by 1 administration of 1.5 – 11 mg of liposomal AmB per kilogram and a total of 98 – 99% of hepatosplenic parasites were eliminated from squirrel monkeys by 3 administrations of 4 mg of liposomal AmB per kilogram.

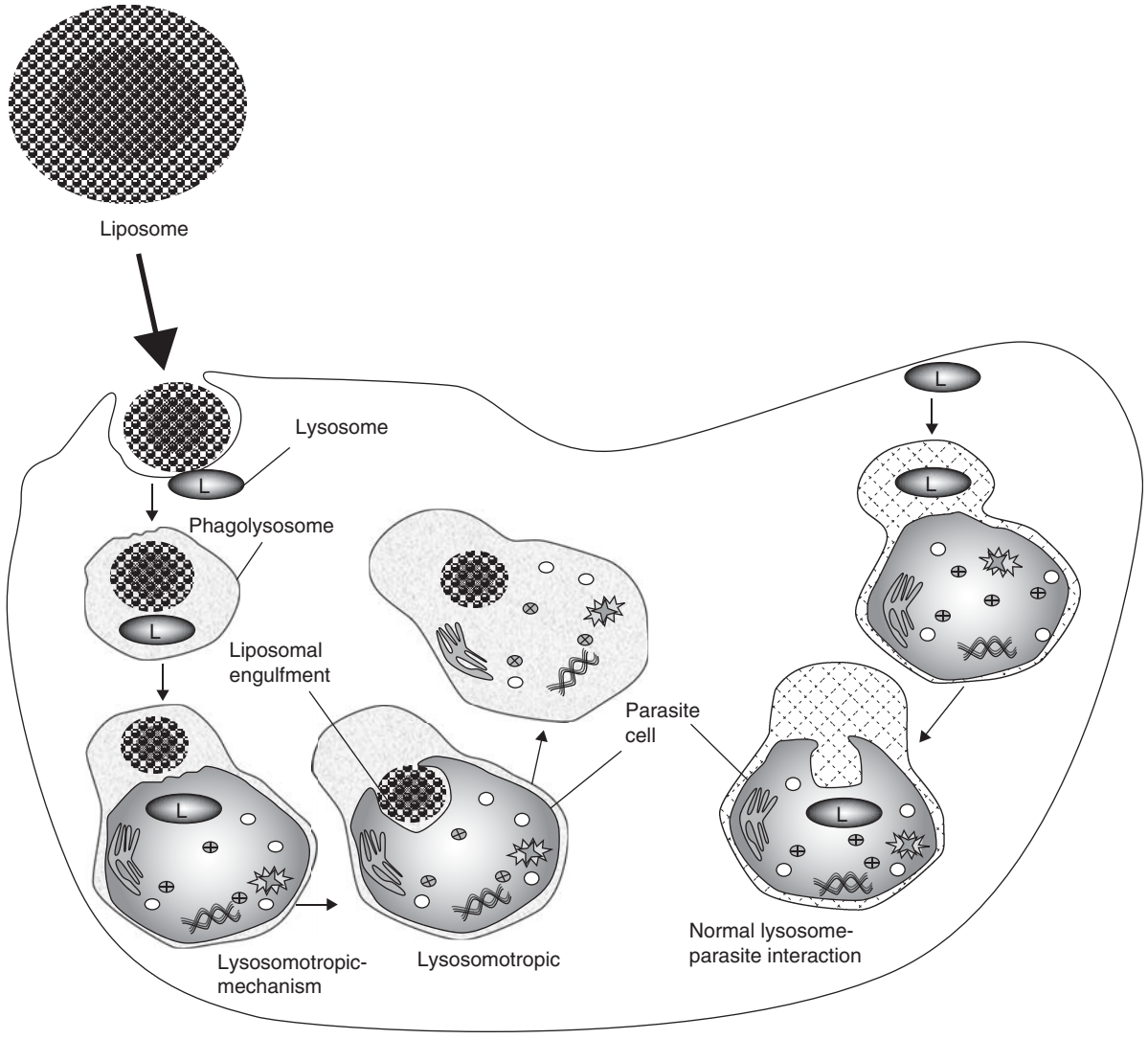


Figure 3. Proposed lysosomotropic–parasitotropic mechanism for delivery of liposome-encapsulated drugs to *Leishmania* parasite within macrophages.

Oliva *et al.* [103] studied the effect of liposomal AmB on dogs naturally infected with *L. infantum*. Dogs that received 3 – 5 administrations of this formulation (at a dose of 3 – 3.3 mg/kg) showed rapid clinical improvement, with regression of lymphadenomegaly and splenomegaly.

In 2000, Cauchetier *et al.* [104] compared the antileishmanial activity of atovaquone-loaded liposomes with that of free drug by determination of median effective doses (ED_{25} and ED_{50}) in a murine model of VL induced by *L. infantum* (4×10^7 promastigotes). On day 21 post-infection (p.i.) the livers were evaluated for parasite burdens using the Stauber method. Parasite suppression of 61.6% was achieved by liposomal atovaquone at a dose of 0.32 mg/kg, whereas only 34.9% parasite suppression was achieved with free drug at a dose of 1.6 mg/kg. Also, median effective doses (ED_{25} value = 0.02 ± 0.01 mg/kg for liposomal drug versus 0.46 ± 0.15 mg/kg for free drug) showed that liposomal drug was 23 times more active than the free drug. ED_{50} for the liposomal form of atovaquone was found to be 0.17 ± 0.05 mg/kg and ED_{50} for free drug was not obtained owing to the formation of a plateau at 33% of parasite suppression in the dose–response curve. However, at a concentration of 1.77 ± 0.35 mg/kg, liposomal drug showed 100% results.

Schettini *et al.* [105] examined multiple dose pharmacokinetics and parasite inhibition (PI) of liposomal formulation of meglumine antimoniate in bone marrow of dogs. Dogs were administered with 4 doses of 6.5 mg/kg (body weight) at intervals of 4 days. Analysis of bone marrow antimony level by electrothermal atomic absorption spectrometry suggested that there was a significant increase in drug concentration from 0.76 (4 days after the first dose) to 2.07 µg/kg (4 days after the

fourth dose). The results suggested that multiple dose treatment with liposomal meglumine antimoniate was successful in improving antimony levels in the bone marrow of dogs infected with *L. chagasi*. Even after 150 days of treatment, significant parasite suppression (> 95.7%) was observed in the lymph nodes, livers and spleens of dogs when immunocytochemical evaluations of the skin, bone marrow, cervical lymph nodes, livers and spleens of dogs were carried out [106].

Schettini *et al.* [107] also provided first direct experimental evidence of passive targeting of liposomes to the bone marrow of dogs infected with VL on reducing the vesicle size from the micrometer to nano size range. An intravenous bolus injection of a new liposomal formulation of meglumine antimoniate of reduced size (400 nm) at a dose of 4.2 mg/kg (body weight) showed threefold enhanced bone marrow antimony level as compared with the drug encapsulated in large liposomes (1200 nm).

5.1.2.1 Cationic liposomes

Cationic liposomes are particles composed of positively charged phospholipids. The charged phospholipids contained in liposomes greatly enhance their binding to the macrophages, which in turn are consequently taken up by these cells. So, a different strategy for targeting VL based on the use of cationic stearylamine-egg phosphatidylcholine-bearing liposomes (SA-PC liposomes) was developed by Dey *et al.* [108]. A single dose of 55 mg of SA-PC liposomes/animal significantly reduced the hepatic parasite burden by 85 and 68% against recent and established experimental VL, respectively. However, the formulation was found to be toxic at high concentrations. The toxicity of SA-PC liposomes for normal murine macrophages was investigated by measuring lactate dehydrogenase activity in the culture medium of liposome-treated macrophages. SA-PC liposomes at 1188 and 396 µg of lipid per milliliter imparted 16.6 and 14.4% toxicity to normal macrophages, respectively, whereas 132 µg of SA-PC liposomes per milliliter showed < 1% toxicity, as determined by the release of lactate dehydrogenase. Lower concentrations of this formulation (66 – 13.2 µg/ml) had no toxic effect on *in vitro* cultured macrophages.

Pal *et al.* [109] also investigated the synergistic activity of combination therapy of SSG entrapped in phosphatidylcholine (PC) and stearylamine (SA) liposomes in both *in vitro* and *in vivo* models of VL. They reported that a single dose of liposomal SSG treatment resulted in almost complete elimination of parasites not only from the liver but also from the spleen of *L. donovani*-infected BALB/c mice. However, an equivalent dose of free SSG or empty PC-SA liposome was only partially effective in clearing the intracellular amastigotes.

A low dose of AmB was also combined with a suboptimum dose of SA-bearing cationic liposomes and the results demonstrated that combination was superior to free AmB and Ambisome in clearing the *L. donovani* parasites from liver and spleen of BALB/c mice, as well as for preventing relapse and reinfection [110].

5.1.2.2 Sterically stabilized liposomes

Sterically stabilized liposomes have been developed to provide long circulation of liposomes and to make the system more stable in biological surroundings. Sterically stabilized liposomes thus avoid their recognition from RES uptake and this 'stealthing' effect makes them long circulatory in nature. Khattab *et al.* [111] reported sorption of silicone-glycol copolymers on the surface of liposomes for their steric stabilization. These sterically stabilized vesicles show enhanced half-life.

Proulx *et al.* [112] coupled polyethylene glycol (PEG) with liposomes to form sterically stabilized liposomes and then studied antileishmanial efficacy of camptothecin (CPT) in free and liposomal form *in vitro* against *L. donovani* promastigotes and *in vivo* in a murine model of VL. Incubation of *L. donovani* promastigotes with free or liposomal CPT inhibited the growth of parasites in a dose-dependent manner. Treatment of infected mice intraperitonealy with free and liposomal CPT significantly reduced the hepatic parasite loads by 43 and 55%, respectively, as compared with the loads of untreated controls. Various liposomal formulations investigated for their antileishmanial activity are summarized in **Table 2**.

5.2 Emulsomes

Emulsomes are pharmaceutical compositions comprising nanoemulsions of particles comprising a lipid core composed of lipid that is in a solid or liquid crystalline phase at at least 25°C, stabilized by at least one phospholipid envelope, for the parenteral, oral, rectal, intranasal, or topical delivery of both fat-soluble and water-soluble drugs [113,114]. These compositions have features that are intermediate between liposomes and oil-in-water emulsions. Particles contain a hydrophobic core, as in standard oil-in-water emulsions, which is surrounded and stabilized by one or more layers or envelopes of phospholipid molecules, as in liposomes. A key feature of these particles is that the core is composed of lipid, which in bulk form is in a solid or liquid crystalline phase, rather than oil in a fluid phase. The internal lipid core is composed of triglycerides (such as tricaprin, trilaurin, trimyristin, tripalmitin and tristearin) and the surrounding envelope consists mainly of phospholipids (such as soybean or egg lecithin, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylserine, phosphatidylcholine, etc.) [114]. Emulsomes offer the advantage of high drug loading of lipophilic drugs such as AmB and also, owing to their colloidal nature, they can be very useful in targeting macrophages for the treatment of VL [115]. Gupta *et al.* [116] developed AmB-bearing emulsomes and evaluated their antileishmanial efficacy. *In vivo* studies on *L. donovani*-infected hamsters showed better results for AmB emulsomes (51.7 ± 5.4% PI) as compared with AmB-Doc (30.4 ± 4.8% PI) in the splenic macrophages, when administered at a dose of 0.5 mg/kg intracardially on alternate days.

Table 2. Various liposomal formulations developed against visceral leishmaniasis.

Drug entrapped	Animal model used	Ref.
Meglumine antimoniate and SSG	*L. donovani*-infected golden hamsters	[99]
AmB	Mice	[100]
Meglumine antimoniate	*L. donovani*-infected dogs	[101]
AmB	*L. donovani*-infected hamsters and monkeys	[102]
AmB	*L. donovani*-infected dogs (naturally infected)	[103]
Atovaquone	*L. infantum*-infected mice	[104]
Meglumine antimoniate	*L. chagasi*-infected dogs (naturally infected)	[105-107]
SSG	*L. donovani*-infected BALB/c mice	[109]
AmB	*L. donovani*-infected BALB/c mice	[110]
Camptothecin	*L. donovani*-infected BALB/c mice	[112]

5.3 Micro/nanopolymeric spheres/particles

Natural polymers such as albumin, cellulose derivatives, gelatin and polysaccharides biodegrade in biological fluids to produce biocompatible or non-toxic products that are removed from the body by normal physiological pathways, and controlled release of loaded drugs can be obtained [117,118]. Microspheres and nanoparticles with different compositions have been widely investigated for drug delivery to macrophages for the treatment of VL.

5.3.1 *Microspheres*

Microspheres are matrix-type particles composed of natural or synthetic polymers in which drugs may be adsorbed at the surface, entrapped or dissolved. The size range of these particles is between 1 and 1000 µm. AmB-loaded microspheres, when administered in the body, resulted in higher concentrations of drug in the liver, spleen and bone marrow, and lower concentrations in the kidneys and lungs [119], thus decreasing the toxicity [120]. Albumin is widely used to prepare microspheres because it is available in pure form and is biodegradable, non-toxic and non-immunogenic [121].

Ordóñez-Gutiérrez *et al.* [122] compared the *in vitro* activity of three AmB aggregation forms (monomeric, dimeric and multi-aggregate) loaded in albumin microspheres with that of multi-aggregate AmB form loaded in poly-lactide-*co*-glycolide (PLGA) microspheres against amastigote and promastigote forms of *L. infantum* using the infected J774 murine macrophage cell lines. Albumin-encapsulated forms did not show any toxicity for murine cells and had lower median effective concentration (EC_{50}) values of 0.003 µg/ml for *L. infantum* amastigotes than free formulations (0.03 µg/ml). None of the three aggregation states of free AmB was able to reduce the promastigote population in the range of concentrations tested. However, encapsulated AmB, both in albumin and in PLGA microspheres, significantly reduced promastigote numbers at drug concentrations >0.2 µg/ml and completely eliminated parasites in the culture medium at the high drug concentration (3.2 µg/ml). The *in vitro* antileishmanial activity of biodegradable starch microspheres covalently linked to primaquine has also been investigated [123].

The antileishmanial activity of albumin microspheres loaded with AmB was tested in *L. infantum*-infected golden hamsters by Dea-Ayuela *et al.* [124]. At a dose of 1 mg/kg administered by the intracardiac route, encapsulated drug showed 88.8 and 87.2% reduction at the early stage of infection (day 32 p.i.) and of 66.7 and 54% reduction at the later stage of infection (day 135 p.i.) in liver and spleen parasite load, respectively, compared with untreated animals, whereas free AmB was found to be inactive. In an another study, Sánchez-Brunete *et al.* [125] compared the albumin microspheres of AmB with AmB-Doc and found that acute toxicity of AmB-loaded albumin microspheres was lower than that of the AmB-Doc in *L. infantum*-infected golden hamsters. At a dose of 2 mg/kg, the AmB-loaded albumin microspheres resulted in higher levels of antileishmanial activity in spleen (72% PI) and liver (90% PI) than AmB-Doc. Even a much higher dose (40 mg/kg (body weight)) of AmB microspheres on intravenous bolus administration did not produce any toxic symptoms.

5.3.2 *Nanoparticles*

Nanoparticles used in drug delivery are generally < 100 nm in size and consist of biodegradable materials such as natural or synthetic polymers, lipids or metals [126]. Drugs can either be incorporated in the matrix of the particle or attached to the particle surface. Espuelas *et al.* [127] studied the antileishmanial activity of AmB loaded into poly(ε-caprolactone) nanospheres coated with poloxamer 188 in an *in vitro* model of macrophages intracellularly infected by two strains of *L. donovani*: a wild strain (WT) and an AmB-resistant strain (AmB^r). AmB-loaded nanoparticles showed enhanced activity compared with free drug only against WT in the macrophage-amastigotes system (50% parasite suppression by AmB-loaded nanoparticles versus 20% parasite suppression by free AmB). The association of AmB with nanospheres increased the activity of free drug against the susceptible promastigotes (IC_{50} between

0.125 and 0.25 for free AmB as compared with IC_{50} of drug associated with carrier between 0.031 and 0.062 µg/ml). However, no differences of activity between free AmB and AmB nanospheres were noticed against resistant promastigotes (IC_{50} between 2 and 4 µg/ml for free drug or drug associated with nanospheres). Primaquine, when loaded in polyisohexylcyanoacrylate [128] and polyalkylcyanoacrylate [129] nanoparticles showed superior antileishmanial activity as compared with free drug. The drug-loaded polyisohexylcyanoacrylate nanoparticles showed a 21-fold increase in antileishmanial activity compared with free drug when evaluated *in vitro* using J774G8 macrophage-like cells infected with *L. donovani*.

Rodrigues *et al.* [130] loaded primaquine in poly(DL-lactide) nanoparticles and administered it intravenously to a BALB/c mice model of *L. donovani* at a dose of 30 mg/kg. The activity of primaquine nanoparticles was found to be 3.3 times that of free drug in terms of amastigote suppression in the liver. The same dose of free drug resulted in 15% weight loss and unloaded nanoparticles were devoid of leishmanicidal activity. Moreover, these nanoparticles were found to be non-toxic [131]. Fouarge *et al.* [132] prepared and characterized polyisohexylcyanoacrylate nanoparticles loaded with dehydroemetine for the treatment of VL in mice and found reproducible preparations with regard to the size and drug adsorption rate. The tissue distribution studies showed that dehydroemetine nanoparticles were rapidly cleared from the bloodstream and were concentrated mainly in the infected tissues. The cardiac toxicity of dehydroemetine was also found to be reduced by its association with nanoparticles.

Pentamidine, a hydrophilic drug, was loaded in polymethacrylate [133] and poly(DL-lactide) [134] nanoparticles and their activity was compared with free pentamidine in a BALB/c mice model of VL induced by *L. infantum*. The results showed that the ED_{50} of bound pentamidine in the form of methacrylate nanoparticles was six times lower than that of free pentamidine (0.17 versus 1.06 mg/kg). Pentamidine-loaded poly(DL-lactide) nanoparticles were 3.3 times more active than free drug (ED_{50} 0.32 versus 1.05 mg/kg). The results suggested that bound pentamidine was more potent than the free drug in the selected model. Another antileishmanial drug, atovaquone, was tested for its efficacy in free and poly (DL-lactide) nanocapsule form in a mice model infected intravenously with 2×10^7 promastigotes of *L. infantum*. The results demonstrated that atovaquone nanocapsules (at a total dose of 3.0 mg/kg) reduced the liver parasite burden by 71.3%, whereas a higher total dose (4.8 mg/kg) of the free drug reduced only 34.4% of the liver parasite burden [135]. Manandhar *et al.* [136] compared the efficacy of a nano form of AmB-Doc with conventional AmB-Doc for the treatment of VL in *L. donovani*-infected hamsters. A dose of 5 mg/(kg day) of nano-AmB-Doc given intraperitoneally for 5 consecutive days resulted in 92.18% PI and 99.18% suppression of parasite replication in spleen, which was found to be superior to conventional AmB-Doc (74.57 and 97.17% PI and suppression of parasite replication, respectively). The results suggested significantly higher effectiveness of nano-AmB-Doc than conventional AmB-Doc.

5.4 Niosomes

Niosomes (NIV) are vesicles that consist mainly of mixtures of cholesterol and non-ionic surfactants such as monoalkyl or dialkyl polyoxyethylene ether or sorbitan esters [137]. Niosomes are the result of self-assembly of hydrated surfactant monomers. Various non-ionic surfactants with a wide variety of structures are a useful alternative to phospholipids in the designing of niosomes [138]. These are the controlled DDSs with higher chemical stability [139]. Moreover, these carriers have been found to reduce systemic toxicity and to enhance site-specific delivery of the entrapped drug. Special production, handling and storage conditions are not required for these carriers. Moreover, relatively low cost of materials makes the industrial manufacturing of niosomes easier [140].

The antileishmanial efficacy of niosomal formulation of sodium stibogluconate (SSG-NIV) was compared with free drug in a laboratory strain (MHOM/ET/67: LV82) and in different clinical isolates of *L. donovani* using BALB/c mice, and the results showed that treatment with SSG-NIV was more effective compared with free SSG against all the strains tested. In SSG-responsive strains, the reduction in liver parasite burdens by SSG-NIV treatment was similar to that caused by free SSG. In non-responsive strains, this formulation showed at least a 95% reduction in liver parasite burden, whereas free SSG reduced only 18% of the liver parasite burden [141]. Mullen *et al.* [142] compared efficacies of SSG-NIV with several formulations of AmB (i.e., Ambisome, Abelcet and Amphocil) in *L. donovani*-infected BALB/c mice. Single-dose treatments of SSG-NIV (296 mg of Sb^V/kg), SSG solution (296 mg of Sb^V/kg) or Ambisome (8 mg of AmB/kg) were equally effective against liver parasites (compared to control values, $p < 0.0005$). SSG-NIV and Ambisome treatment also significantly suppressed parasites in bone marrow and spleen ($p < 0.005$), with SSG-NIV treatment being more suppressive (> 98% suppression in all three sites, that is, liver, spleen and bone marrow), whereas free SSG failed to suppress spleen or bone marrow parasites. This study suggested that the developed formulation was more effective in treating VL compared with free drug or new AmB formulations; however, the formulation did not protect against reinfection [143].

Moreover, Williams *et al.* [144] had previously obtained more prominent results (i.e., 74 ± 10, 99 ± 1 and 38 ± 8% suppression of liver, spleen and bone marrow parasite burdens, respectively, in the BALB/c mice model of VL) using large SSG-NIV (mean diameter > 800 nm). Niosomes loaded with SSG eliminated parasites not only from the liver but also from the spleen and bone marrow of the *L. donovani*-infected murine model of VL. Three different types of niosome prepared with different non-ionic surfactants containing 30 mol% cholesterol did not show significant difference in

the activity. Both negatively charged and neutral vesicles were found to be equally valuable [145].

Nieto *et al.* [146] determined the pharmacokinetics and toxicities (in dogs) and antileishmanial efficacies (in *L. donovani*-infected BALB/c mice) of NIV and NIV-dextran forms of SSG after administration of a single intravenous dose. The NIV-dextran form significantly modified the pharmacokinetics of the drug, whereas the free drug as well as the NIV form showed similar pharmacokinetics. The antileishmanial activity of the drug was appreciably enhanced in the NIV form and even more in niosomes covered with dextran. Leishman-Donovan units (LDUs; where LDU is the number of amastigotes per 1000 host cell nuclei × organ weight (in grams)) were calculated for the liver and spleen and the results showed that on treatment with SSG-NIV-dextran, at a dose of 33 mg/kg, parasite burdens were found to be 0.48 ± 0.30 and 0.70 ± 0.30 LDUs in the spleen and liver, respectively, whereas at even higher dose (222 mg/kg), the parasite burdens were found to be higher in the cases of SSG-NIV (0.85 ± 0.60 and 1.77 ± 1.36 LDUs in spleen and liver, respectively) and free SSG (1.78 ± 1.30 and 2.32 ± 2.05 LDUs in spleen and liver, respectively). No signs of toxicity were found in mice after administration of these formulations, although short-term toxicity in dogs was demonstrated by the development of chills, diarrhea and hepatic dysfunction, which was cleared at 24 h postdosing.

Baillie *et al.* [147] also encapsulated SSG in NIV and tested its activity in a murine model of VL. SSG-NIV was found to be more active than free drug. Williams *et al.* [148] also compared the effect of different surfactants on the production and *in vivo* efficacy of niosomes and reported that the decaethylene glycol mono *n*-hexadecyl ether paromomycin NIV were more effective than hexaethylene glycol mono *n*-hexadecyl ether paromomycin vesicles in terms of PI in liver and spleen of the *L. donovani*-infected murine model of VL. Both types of paromomycin-loaded NIV were more effective than free drug against liver and spleen parasites, but neither the NIV nor free forms of paromomycin caused significant suppression of bone-marrow parasites.

Another approach, solubilization of AmB in cyclodextrins as an inclusion complex and then further loading of solubilized AmB in niosomes, was developed by Mullen *et al.* [149]. This formulation (showed 79 ± 4, 89 ± 2 and 74 ± 7% parasite suppression in spleen, liver and bone marrow, respectively) offered significantly higher activity than Abelcet (showed 62 ± 10, 90 ± 3 and 26 ± 11% parasite suppression in spleen, liver and bone marrow, respectively) and Fungizone (omitted owing to toxicity), but lower activity than Ambisome (showed 86 ± 3, 99.5 ± 0.2 and 73 ± 6% parasite suppression in spleen, liver and bone marrow, respectively) and Amphocil (showed 96 ± 2, 100 ± 0 and 77 ± 6% parasite suppression in spleen, liver and bone marrow, respectively) in eliminating parasites from the spleen, liver and bone marrow in experimental VL at the same AmB dose (2.5 mg/kg).

5.5 Polymer conjugates

Polymer–drug conjugates have been used for targeting to macrophages and have already shown potential in antileishmanial chemotherapy, so a different strategy was developed for targeting macrophages, which also modified the biodistribution of antileishmanial drugs (such as AmB) that were otherwise toxic for mammal cells when aggregated with water [150].

AmB when conjugated with *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer through a degradable GlyPheLeuGly linker showed 99.6 and 93.8% inhibition in hepatic parasite burden at a dose of 3 and 1 mg/kg (body weight), respectively, when administered intravenously to *L. donovani*-infected BALB/c mice. Ambisome was taken for comparison, which showed 99.9% PI at the same doses [151]. Koczan *et al.* [152] coupled methotrexate (MTX) with different synthetically branched polypeptides such as poly [Lys(DL-Ala*m*)](AK), poly[Lys(Ser_i-DL-Ala*m*)](SAK), poly [Lys(DL-Ala*m*-Leu_i)](ALK) and poly[Lys(Glu_i-DL-Ala*m*)] (EAK). They studied the effect of this conjugation on *L. donovani* infection in BALB/c mice. Out of these conjugates, MTX-ALK produced the most encouraging data, with 95% PI in liver as compared with free MTX (42% PI) when 5 injections (100 µg of MTX/injection) were administered intraperitonealy. Moreover, they also stated that the covalent bond between the carrier and the drug is crucial for its activity.

In another study by Nan *et al.* [153] an aminoquinoline analogue, NPC1161, was evaluated for its antileishmanial activity by conjugating it with HPMA copolymer. Conjugates of HPMA copolymer with NPC1161, containing *N*-acetyl-mannosamine (ManN) in the side chains, were synthesized and *in vivo* studies in *L. donovani*-infected BALB/c mice showed that administration of an intravenous drug dose of 1 mg/kg in the form of HPMA copolymer-NPC1161 conjugates with 5 mol% or higher ManN content were significantly more effective (showed 67 – 80% PI) than HPMA copolymer-NPC1161 conjugates (showed 47% PI). HPMA copolymers containing ManN in the side chains could potentially reduce the toxicity and increase the efficacy of antileishmanial drugs for the treatment of VL. The above-mentioned DDSs are summarized in **Table 3**.

6. Receptor-mediated active targeting to macrophages for the treatment of visceral leishmaniasis

The process of active targeting exploits modification or manipulation of the natural distribution pattern of the drug carrier using some exogenous means, so that it can be identified by particular cell lines. Macrophages possess various receptors such as Fc receptors, complement, fibronectin lipoprotein, mannosyl, galactosyl and many other receptors [154]. These macrophage surface receptors determine the control of activities such as activation, recognition, endocytosis, secretion, and so on [155]. Likewise, for diseases of microbial

Table 3. Drug delivery systems other than liposomes for targeting visceral leishmaniasis.

Carrier system	Drug encapsulated	Properties	Diseased model used	Ref.
Emulsomes	AmB	Trilaurin-based particles stabilized by soya phosphatidylcholine having size 0.26 ± 0.02 μm and entrapment efficiency 88.6 ± 6.21%, dose given – 0.5 mg/kg i.c.	*L. donovani*-infected Syrian golden hamsters	[116]
Polymeric particles	AmB	Albumin and PLGA microspheres with EC_{50} of 0.0037 ± 0.00232 and 0.033 ± 0.0115 μg/ml, respectively	*L. infantum*-infected J774 murine macrophage cell lines	[122]
	AmB	Albumin microspheres, dose given – 1 mg/kg given i.c.	*L. infantum*-infected golden hamsters	[124]
	AmB	Albumin microspheres, 1 ± 0.7 μm in size, dose given – up to 40 mg/kg i.v.	*L. infantum*-infected golden hamsters	[125]
	AmB	Poly(ε-caprolactone) nanospheres coated with poloxamer 188, 358 ± 62 nm in size	*L. donovani*-infected macrophages harvested from CDI mice	[127]
	Primaquine	Polyisohexylcyanoacrylate nanoparticles, 200 – 250 nm in size, 80 – 90% drug binding, formulation given i.v.	NMRI mice model of VL	[128]
	Primaquine	Polyalkylcyanoacrylate nanoparticles showing a 21-fold increase in ED_{50} as compared with free drug	*L. donovani*-infected J774G8 macrophage cell lines	[129]
	Primaquine	Poly(DL-lactide) nanoparticles, dose given – 30 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[130]
	Dehydroemetine	Polyisohexylcyanoacrylate nanoparticles, 246 nm in size, 90% drug entrapment, dose given – 10 mg/kg i.v.	NMRI mice model of VL	[132]
	Pentamidine	Polymethacrylate nanoparticles with ED_{50} of 0.17 mg/kg and ED_{90} of 1 mg/kg i.v.	*L. infantum*-infected BALB/c mice	[133]
	Pentamidine	Poly(DL-lactide) nanoparticles with ED_{50} of 0.32 mg/kg i.v.	*L. infantum*-infected BALB/c mice	[134]
	Atovaquone	Poly(DL-lactide) nanocapsules, dose given – 3 mg/kg i.v.	*L. infantum*-infected mice	[135]
	AmB	Nanoparticles of size range 10 – 20 nm, dose given – 5 mg/(kg day) i.p.	*L. donovani*-infected hamsters	[136]
Niosomes	SSG	Mono-*n*-hexadecyl ether tetraethylene glycol, cholesterol and dicetyl phosphate (molar ratio 3:3:1) containing vesicles, dose given – 300 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[141]
	SSG	Mono-*n*-hexadecyl ether tetraethylene glycol, cholesterol and dicetyl phosphate (molar ratio 3:3:1) containing vesicles, dose given – 296 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[142]
	SSG	Parasite suppression of 74 ± 10, 99 ± 1 and 38 ± 8% in liver, spleen and bone marrow, respectively, was achieved using vesicles of mean diameter > 800 nm	BALB/c mouse model of VL	[144]
	SSG	High level of SSG in liver was attained after i.v. administration	*L. donovani*-infected murine model of VL	[145]
	SSG	Mono-*n*-hexadecyl ether tetraethylene glycol, cholesterol and dicetyl phosphate (molar ratio 3:3:1) containing vesicles, 526 ± 20 nm in size, entrapment efficiency 6%, dose given – 222 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[146]

i.c.: Intracardiac; i.p.: Intraperitoneal; i.v.: Intravenous.

Table 3. Drug delivery systems other than liposomes for targeting visceral leishmaniasis (continued).

Carrier system	Drug encapsulated	Properties	Diseased model used	Ref.
	SSG	High drug levels in the infected RES were achieved with SSG-entrapped niosomes	Murine model of VL	[147]
	Paromomycin	Vesicles containing different surfactants such as decaethylene glycol mono-*n*-hexadecyl ether and hexaethylene glycol mono-*n*-hexadecyl ether	*L. donovani*-infected BALB/c mice	[148]
	AmB	Mono-*n*-hexadecyl ether tetraethylene glycol, cholesterol, and dicetyl phosphate (molar ratio 3:3:1) containing vesicles, 240 – 530 nm in size, dose given – 2.5 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[149]
Polymer conjugates	AmB	*N*-(2-hydroxypropyl) methacrylamide (HPMA)-AmB copolymer conjugates, dose given – 3 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[151]
	MTX	Conjugates of methotrexate with different cationic and amphoteric synthetic polypeptides containing poly[L-Lys] backbone	*L. donovani*-infected BALB/c mice	[152]
	NPC1161	Conjugates of NPC1161 with HPMA copolymer containing *N*-acetylmannosamine (ManN) in the side chains, dose given – 1 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[153]
Ionic amphiphilic biovector	AmB	Particles comprising anionic lipid dipalmitoyl phosphatidyl glycerol included in a cationic crosslinked polysaccharide matrix, 100 nm in size, 20% drug entrapment, administered at a dose of 5 mg/kg i.v.	*L. donovani*-infected BALB/c mice	[201]

i.c.: Intracardiac; i.p.: Intraperitoneal; i.v.: Intravenous.

etiology such as VL, the intracellular localization of the pathogens necessitates the administration of relatively high doses of the cytotoxic drugs for the effective killing of the pathogens, thereby causing the side effects. The rational approach to the problem requires that drugs should be targeted to the macrophages in such a way that the interaction of the free drug with non-target tissues could be minimized [115]. Many approaches for targeting the drugs to the macrophages have been developed, which are largely represented by colloidal carriers. Although these carriers show natural affinity towards the macrophages and are passively targeted to them, inclusion of the macrophage receptor(s)-specific ligands may significantly enhance the rate and extent of their uptake by the macrophages (**Figure 4**). The macrophage-specific ligands such as tuftsin and mannose residues have been incorporated into liposomes to enhance their active uptake by macrophages.

Tuftsin is a basic tetrapeptide (Thr-Lys-Pro-Arg) that is found to reveal several biological functions associated with the immune system. It is generated in the body from a specific cytophilic fraction of the protein (leukokinin) through a two-step enzymatic processing mechanism [156,157]. The tetrapeptide enhances the phagocytic activity of monocytes and macrophages [158-160]. Specific binding sites for tuftsin have been revealed to exist on macrophages [161]. Tuftsin exclusively binds to macrophages and potentiates their natural killer activity against pathogens [162]. This makes tuftsin an attractive candidate to be used as a ligand for targeting the drugs to various macrophage-related diseases. Tuftsin-based targeting of SSG liposomes (composed of egg phosphatidylcholine and cholesterol) was studied by Guru *et al.* [163] in *L. donovani* infected hamsters for VL. In the study, free drug showed 90% PI (on day 28 post-treatment) in spleen at a dose of 10 mg/(kg day) administered for 5 consecutive days. On reducing the dose of free drug to 500 and 250 µg/(kg day), the extent of this inhibition was reduced significantly to 35.8 ± 6.6 and 51.9 ± 4.7%, respectively, on day 7 post-treatment. At a dose of 500 µg/(kg day), tuftsin-bearing liposomes showed much better results (77.6 ± 1.8 and 85.0 ± 3.1% PI in spleen on day 7 and 28 post-treatment, respectively) than those of tuftsin-free liposomes (65.0 ± 6.1 and 61.5 ± 1.3% PI in spleen on days 7 and 28 post-treatment, respectively); but this difference was reduced by reducing the drug dose to 250 µg/(kg day). At a dose of 250 µg/(kg day), tuftsin-bearing liposomes showed 86.5 ± 4.5 and 92.1 ± 2.7% PI, whereas tuftsin-free liposomes showed 74.8 ± 5.1 and 80.5 ± 11.6% PI on days 7 and 28 post-treatment, respectively. These findings indicated that encapsulation of SSG in tuftsin-bearing liposomes significantly enhanced the drug efficacy against *L. donovani* infection.

The antileishmanial activity of AmB was also enhanced by encapsulating the drug in liposomes. At doses of 0.5 and

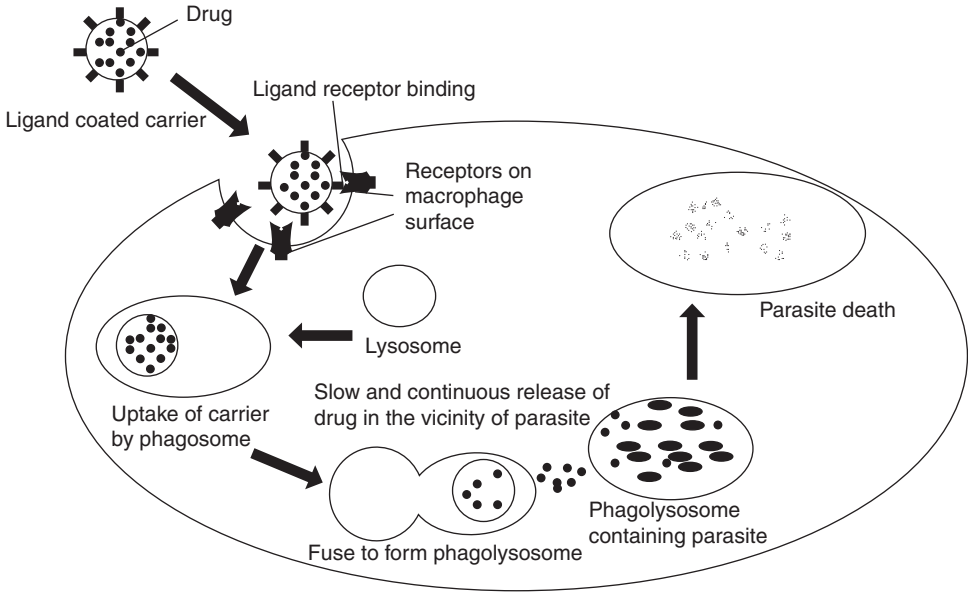


Figure 4. Schematic representation of receptor-mediated active targeting to macrophages for the treatment of visceral leishmaniasis.

1 mg/ml free drug resulted in only 38 and 39% PI, respectively, in spleen of *L. donovani*-infected hamsters, whereas the liposomal drug resulted in 60 and 71% PI, respectively, at the same doses. This antileishmanial effect of the liposomal AmB was increased further by grafting tuftsin on the liposomal surface. At the same doses, that is, 0.5 and 1 mg/ml, the tuftsin-grafted liposomes resulted in 82 and 81% PI, respectively. The tissue distribution studies also showed the higher and faster uptake of tuftsin-grafted liposomes from the circulation as compared with non-grafted liposomes. Almost all the grafted liposomes were cleared within 1 h of administration [164].

O-palmitoyl mannan (OPM) has also been widely used for active targeting to macrophages. Mannosylated liposomes were found to be more convincing in delivering antileishmanial drugs to macrophages. Selective delivery of liposomes was performed by targeting the mannose receptors on the surface of macrophages [165-167]. Mannose-grafted pentamidine isethionate liposomes were found to be the most effective, with 85.1% reduction in splenic parasite load as compared with glucose-grafted liposomes (showed 65.9% reduction in splenic parasite load), galactose-grafted liposomes (showed 45.1% reduction in splenic parasite load), uncoated liposomes (showed 46.6% reduction in splenic parasite load) and free drug (showed 18.5% reduction in splenic parasite load), when tested in *L. donovani*-infected hamsters [168].

Kole *et al.* [169] found that mannosylated liposomes of doxorubicin were more effective than liposomal or free doxorubicin in the treatment of VL in *L. donovani*-infected BALB/c mice. They combined doxorubicin with mannosylated liposomes and IFN-α. The combined chemotherapy resulted in complete elimination of splenic parasite burden. Further, they analyzed mRNA levels of infected spleen cells and suggested that targeted drug delivery together with IFN-α also resulted in reduced levels of IL-4, increased levels of IL-12 and inducible nitric oxide synthase. Such a combination chemotherapy was proved to be a capable substitute for the cure of VL. Mitra *et al.* [170] compared the antileishmanial property of a benzyl derivative of a new antibiotic MT81 (Bz_2MT81) in free, liposome-intercalated and mannose-grafted liposome-intercalated forms in *L. donovani*-infected hamsters. The results showed that at a dose equivalent to 7.5 mg/kg (body weight) injected subcutaneously in the form of mannose-grafted liposomes for 15 days at an interval of 3 days, the splenic PI was found to be 79.1%, whereas free and liposomal drug forms were found to be less effective in reducing the parasite load in spleen (49.8 and 55.1% parasite suppression was shown by free and liposomal drug, respectively). AmB was formulated in uncoated and OPM-coated trilaurin emulsomes. In terms of reduction in splenic parasite burden in *L. donovani*-infected hamsters, OPM-coated AmB emulsomes were found to be more efficient (showed 73.7 ± 6.7% PI) as compared with plain AmB emulsomes (showed 51.7 ± 5.4% PI) and AmB-Doc (showed 30.4 ± 4.8% PI) when administered at a dose of 0.5 mg/kg intracardially on alternate days [116].

Liposomes appended with antibodies or their fragments on their surfaces are known as immunoliposomes. Many approaches in the field of drug, enzyme and gene delivery have been attempted to achieve targetability using immunoliposomes. Immunoliposomes have been used extensively as a drug delivery strategy towards macrophages for the treatment of VL. Recent developments in liposomal technology have made it feasible to investigate therapeutic applications

Table 4. Various ligand-grafted carrier systems developed against visceral leishmaniasis.

Carrier system	Drug encapsulated	Animal model used	Ref.
OPM-coated emulsomes	AmB	*L. donovani*-infected hamsters	[116]
Tuftsin-bearing liposomes	SSG	*L. donovani*-infected golden hamsters	[163]
Tuftsin-bearing liposomes	AmB	*L. donovani*-infected golden hamsters	[164]
Mannose-grafted liposomes	Pentamidine isethionate	*L. donovani*-infected hamsters	[168]
Mannosylated liposomes	Doxorubicin	*L. donovani*-infected BALB/c mice	[169]
Mannosylated liposomes	MT81	*L. donovani*-infected golden hamsters	[170]
Immunoliposomes	Doxorubicin	*L. donovani*-infected BALB/c mice	[171]
Mannose-coated lipid nanospheres	AmB	*L. donovani*-infected BALB/c mice	[173]

involving site-specific delivery mediated through antibodies. Anti-target antibody (anchored on the liposome surface) having specific avidity to target could direct liposomes to the desired target. Active targeting of doxorubicin to *L. donovani*-infected BALB/c mice was studied by Mukherjee *et al.* [171] by incorporating it in immunoliposomes prepared by grafting $F(ab)'_2$ of anti-51-kDa antibody onto the liposomal surface. The results showed that, at a dose of 250 mg/(kg day) administered for 4 consecutive days, there was complete elimination of splenic parasite burden by doxorubicin immunoliposomes as compared with a similar dose of free (showed 45% PI) and liposomal drug (showed 84% PI). The results also showed reduced toxicity of liposomal formulations. In another approach liposomes grafted with IgG (immunoliposomes) resulted in superior efficacy than free IgG and plain liposomes in clearing *L. donovani* parasites from the macrophages, owing to their increased uptake by the Fc receptors in macrophages. On incubation (at 37°C for 5 min) of liposomal IgG with macrophages infected with different strains of *L. donovani* (UR6, AG83 and GE1 strains), the induced macrophage activation suppressed the parasite burden of different strains to an extent of ~ 60, 50 and 45%, respectively [172].

Recently, Veerareddy *et al.* [173] 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 Fungizone was also administered in separate mice as control groups. On measuring the parasite burden, it was found that mannose-anchored AmB lipid nanospheres reduced 95 and 94%, AmB lipid nanospheres reduced 90 and 85% and Fungizone reduced 82 and 69% of parasite burden in the liver and spleen, respectively. Various ligand-grafted carrier systems for the treatment of VL are summarized in **Table 4**.

7. Natural plant products as antileishmanial agents

The development of various drugs for the treatment of VL has emerged as an advantage to drug discovery; but the emerging drug resistance in various species of *Leishmania* parasite has stimulated the interest of researchers in the development of compounds with even more significant antileishmanial activity and reduced side effects. Screening of various natural products has emerged as a progressive step in this regard. The need for a valuable antileishmanial drug has renewed interest in the study of medicinal plants as a source of new chemotherapeutic compounds with superior efficacy and reduced side effects. Many natural plant products reveal antileishmanial properties and are highly discriminatory in their mode of action.

7.1 Plant metabolites with antileishmanial activity

Plant products with leishmanicidal activity include quinones, alkaloids, terpenes, saponins, phenolic derivatives and other metabolites [174,175]. The new compounds showing antileishmanial activity are some of the alkaloids such as benzoquinolizidine alkaloids [176] and manzamine alkaloids [177], terpenes such as diterpenoids [178], triterpenoids [179] and sesquiterpenes [180], and phenolics such as neolignans [181] or naphthoquinones [182]. Another group of natural products with leishmanicidal properties are chalcones [183-185]. The main sources of new compounds with antileishmanial activity are metabolites derived from plants. Various metabolites extracted from natural plants and investigated for antileishmanial activity are summarized in **Tables 5 – 9**.

7.2 Plant extracts with antileishmanial activity

The variety of natural plant products and herbal remedies offers a more reliable therapy for the treatment of VL. Recently, extracts of various natural plant products have been studied to evaluate their antileishmanial activity. Various plant extracts evaluated for their antileishmanial activity are summarized in **Table 10**.

7.3 Various carriers entrapping natural plant products

Most of the literature emphasizes selection of new antileishmanial compounds from the natural products rather than optimization of the activity of already known compounds. Unfortunately, most of these compounds failed in the requirements of drug development owing to their undesirable properties, such as high toxicity, poor solubility, low

Table 5. Quinones having antileishmanial activity.

Category	Plant metabolites and their source	Active against	Ref.
Bis-naphthoquinone	Diospyrin isolated from the bark of *Diospyros montana*	*L. donovani* promastigotes at MIC of 1 μg/ml	[202]
		Topoisomerase I enzyme in *L. donovani*	[203]
Hydroxynaphthoquinone	Hydroxylated derivative of diospyrin isolated from the bark of *D. montana*	*L. donovani* amastigotes at a concentration of 3 μM	[204]
Naphthoquinone	Plumbagin isolated from *Plumbago* spp.	*L. donovani* amastigotes at IC_{50} of 0.42 μg/ml	[205]
	Plumbagin isolated from the bark of *Pera benensis*	*L. donovani* promastigotes at IC_{90} of 5 μg/ml	[206]
	3,3′-biplumbagin and 8,8′-biplumbagin isolated from the bark of *P. benensis*	*L. donovani* promastigotes at IC_{90} of 50 and 5 μg/ml, respectively	[206]
Prenylated hydroxynaphthoquinone	Lapachol obtained from *Tecoma* spp.	*L. donovani* amastigotes	[207]
Tetralones	4-hydroxy-1-tetralone isolated from the bark of *Ampelocera edentula*	*L. donovani* promastigotes at IC_{90} of 10 μg/ml	[208]
Prenylated dihydroquinone	Hydropiperone isolated from *Peperomia galioides*	*L. donovani* promastigotes at a concentration of 100 μg/ml	[209]
Anthraquinones	Aloe-emodin isolated from the aerial parts of *Stephania dinklagei*	*L. donovani* amastigotes at IC_{50} of 90 μM and promastigotes at IC_{50} of 185.1 μM	[210]

MIC: Minimum inhibitory concentration.

bioavailability and less than usual efficiency at modest doses [185]. However, several studies have been carried out to minimize the side effects of the existing compounds and to enhance their antileishmanial efficacy. Various approaches include the entrapment of various antileishmanial natural plant products in different carrier systems to enhance their effectiveness (**Table 11**). Bassic acid, an unsaturated triterpene acid isolated from *Mimusops elangii*, was tested both *in vitro* and *in vivo* for its antileishmanial properties. The *in vivo* activity was evaluated in the hamster model of VL, both in free form as well as in the form of two different delivery systems, *viz* microemulsions and poly(DL-lactide) nanoparticles. At a dose of 2 mg/kg (body weight) injected subcutaneously, the parasite reduction in the spleen was found to be 45, 62 and 78% in free, microemulsion and nanoparticle forms, respectively. Having been proved to be non-hepatotoxic and non-nephrotoxic, the nanoparticulate form of bassic acid was considered to be better than the microemulsion form [186].

Various indigenous compounds, namely harmine [187], arjunglucoside I [188] and quercetin [189], have been investigated for antileishmanial activity by using different delivery systems. Harmine, a beta-carboline amine alkaloid isolated from *Peganum harmala*, was encapsulated in different carriers, namely liposomes, niosomes and nanoparticles. The alkaloid injected subcutaneously at an equivalent dose of 1.5 mg/kg (body weight) in *L. donovani*-infected hamsters, for a total of 6 doses in 15 days, was found to reduce splenic parasite burden by ~ 40, 60, 70 and 80% in free, liposomal, niosomal and nanoparticular forms, respectively. It was also found that the toxicity of the compound was reduced in these carrier forms in the same order as their efficacy [187]. Arjunglucoside I was incorporated in ultra-low-sized nanogel (~ 90 nm in diameter) composed of crosslinked random copolymer of *N*-isopropylacrylamide (NIPAAM) and *N*-vinyl pyrrolidone (NVP) and its antileishmanial efficacy was compared with free form and arjunglucoside I-encapsulated hydrophobic poly(DL-lactide) nanoparticles (~ 250 nm in diameter) in the hamster model of VL. The free form reduced the splenic parasite load by 38% only, whereas copolymeric nanogels and polymeric nanoparticles reduced the parasitic load by 79 and 75%, respectively. Both the nanocarriers reduced hepatotoxicity and nephrotoxicity almost to the same extent [188]. Quercetin was encapsulated in different carrier forms, namely liposomes, niosomes, microspheres and nanoparticles, and its antileishmanial efficacy in various delivery modes was tested in the hamster model of VL. At equivalent quercetin concentration, the nanocapsulated quercetin was found to be the most potent at reducing the parasite burden in the spleen as well as in reducing hepato and nephrotoxicity as compared with free drug or other carrier forms of drug [189].

Amarogentin [190], a plant glycoside isolated from the Indian medicinal plant *Swertia chirata*, was evaluated for antileishmanial efficacy in free and two different vesicular forms, liposomes and niosomes, in *L. donovani*-infected hamsters. At a dose of 2.5 mg/kg injected subcutaneously every 3 days, free amarogentin reduced splenic parasite load by only 34%, whereas liposomal and niosomal forms reduced splenic parasite load by 69 and 90%, respectively. Another plant glycoside, bacopasaponin C [191], isolated from *Bacopa monniera*, was also tested for antileishmanial properties both

Table 6. Alkaloids having antileishmanial activity.

Category	Plant metabolites and their source	Active against	Ref.
Quinoline and isoquinoline analogues	Isoguattouregidine isolated from the bark of *Guatteria foliosa*	*L. donovani* at a concentration of 100 μg/ml	[211]
	Anonaine obtained from the trunk bark and roots of *Annona spinescens*	*L. donovani* promastigotes	[212]
	Liriodenine obtained from the trunk bark and roots of *A. spinescens*	*L. donovani* promastigotes at IC_{100} of 100 μg/ml	[212]
	Liriodenine obtained from the stem bark of *Rollinia emarginata*	*L. donovani* promastigotes at IC_{100} of 5 μg/ml	[213]
	Daphnandrine isolated from *Albertisia papuana*	*L. donovani* promastigotes at IC_{100} of 50 μg/ml	[214]
	Obaberine isolated from *Pseudoxandra sclerocarpa*	*L. donovani* promastigotes at IC_{100} of 50 μg/ml	[214]
	Limacine isolated from *Caryomene olivascens*	*L. donovani* promastigotes at IC_{100} of 50 μg/ml	[214]
	Gyrocarpine isolated from *Gyrocarpus americanus*	*L. donovani* promastigotes at IC_{100} of 50 μg/ml and at a concentration of 10 μg/ml	[214,215]
	Isotetradrin isolated from *Limaciopsis loangensis*	*L. donovani* promastigotes at a concentration of 10 μg/ml	[215]
	Isotetradrin isolated from the stem bark of *Guatteria boliviana*	*L. donovani* promastigotes	[216]
	Chimanine D isolated from the leaves of *Galipea longiflora*	*L. donovani*	[217]
	2-*n*-propylquinoline isolated from the leaves of *G. longiflora*	*L. donovani* at a concentration of 0.54 mmol/kg	[218,219]
Indole analogues	Harmine, Pleiocarpine and Buchtienine isolated from the stem bark and leaves of *Kopsia griffithii*	*L. donovani* promastigotes	[220]
Steroidal alkaloids	Sarachine isolated from the leaves of *Saracha punctata*	*L. donovani* promastigotes at a concentration of 10 μg/ml	[221]
	Holamine, 15-α-Hydroxyholamine, Holacurtine and *N*-desmethylholacurtine obtained from the leaves of *Holarrhena curtisii*	*L. donovani* promastigotes	[222]
Other alkaloids	Piperine and Benzoxazol-2(3*H*)-one obtained from the leaves of *Acanthus illicifolius*	*L. donovani* promastigotes	[223,224]
	Peganine hydrochloride isolated from the seeds of *Peganum harmala*	*L. donovani* amastigotes at IC_{90} of 85 μg/ml and IC_{50} of 41 μg/ml and promastigotes at IC_{90} of 75 μg/ml and IC_{50} of 38 μg/ml	[225]
		Topoisomerase I enzyme in *L. donovani*	[225,226]
	Cadambine acid isolated from *Nauclea diderrichii*	*L. infantum* amastigotes at IC_{50} of 1 μM	[227]

in free form and in different delivery modes (i.e., niosomes, microspheres, nanoparticles and liposomes) in *L. donovani*-infected hamsters. The results illustrated that, at equivalent dose of 1.75 mg/kg (body weight), administered every third day for a total of 6 doses in 15 days, bacopasaponin C in all the delivery modules was found to be very active. Raay *et al.* [192] intercalated piperine in liposomes and mannose-coated liposomes and evaluated their antileishmanial activity in *L. donovani*-infected hamsters. At a dose of 6 mg/kg (body weight) administered every fourth day for a total of 4 doses in 12 days, splenic parasite load was reduced to 90% with mannose-coated liposomal piperine as compared with liposomal piperine (showed 77% PI) or free piperine (showed 29% PI). The toxicity of piperine was also reduced when it was administered in mannosylated liposomal form.

In another study, Veerareddy *et al.* [193] investigated piperine for the treatment of VL by formulating it in lipid nanospheres. A single dose of 5 mg/kg piperine, lipid

Table 7. Terpenes having antileishmanial activity.

Category	Plant metabolites and their source	Active against	Ref.
Iridoids	Arbortristosides A isolated from the seeds of *Nyctanthes arbortristis*	*L. donovani* amastigotes at concentrations of 10 mg/kg (i.p.) and 100 mg/kg (oral)	[228]
	Arbortristosides B, Arbortristosides C and 6-β-hydroxyloganin isolated from the seeds of *N. arbortristis*	*L. donovani* amastigotes	[228]
	Picroside I and Kutkoside obtained from the roots and rhizomes of *Picrorhiza kurroa*	*L. donovani* promastigotes	[229]
	Amarogentin isolated from *Swertia chirata*	Topoisomerase I enzyme in *L. donovani* at a concentration higher than 60 μM	[230]
Monoterpenes	Espintanol isolated from the bark of *Oxandra espintana*	*L. donovani* promastigotes	[231]
	Grifolin and Piperogalin obtained from *Peperomia galoides*	*L. donovani* promastigotes at a concentration of 100 μg/ml	[232]
Sesquiterpenes	Dehydrozaluzanin C isolated from the leaves of *Munnozia maronii*	*L. donovani* promastigotes at concentrations between 2.5 and 10 μg/ml	[233]
	Kudtriol obtained from the aerial parts of *Jasonia glutinosa*	*L. donovani* promastigotes at a concentration of 250 μg/ml	[234]
Diterpenes	Jatrogrossidione and Jatrophone isolated from *Euphorbiaceae* spp.	*L. chagasi* promastigotes at IC_{100} of 0.75 and 5 μg/ml, respectively	[235]
	Dehydropinifolic acid 15-monomethyl ester obtained from the stem bark of *Polyalthia macropoda*	*L. donovani* promastigotes	[236]
	Ribenol isolated from *Sideritis varoi*	*L. donovani* amastigotes and promastigotes	[237]
	6-β-hydroxyrosenonolactone isolated from the bark of *Holarrhena floribunda*	*L. donovani* amastigotes and promastigotes	[238]
Triterpenes	(24*Z*)-3-oxotirucalla-7,24-dien-26-oic acid and epi-oleanolic acid obtained from the leaves of *Celaenododendron mexicanum*	*L. donovani* promastigotes at IC_{50} of 13.7 and 18.8 μM, respectively	[239]
	Simalikalactone D and 15-β-heptylchaparrinone obtained from *Simaroubaceae* spp.	*L. donovani* promastigotes	[240]
Saponins	α-hederin and β-hederin obtained from the leaves of *Hedera helix*	*L. infantum* amastigotes and promastigotes	[241]
	Hederecolchiside A_1 obtained from *Hedera colchica*	*L. infantum* amastigotes and promastigotes	[241]
	Hederagenin obtained from the leaves of *H. helix*	*L. infantum* amastigotes and promastigotes	[242]
	Racemoside A obtained from the fruits of *Asparagus racemosus*	*L. donovani* amastigotes at IC_{50} of 1.15 and 1.31 μg/ml and promastigotes at IC_{50} of 0.17 and 0.16 μg/ml (in AG83 and GE1F8R strain, respectively)	[243]
	PX-6518 obtained from the leaves of *Maesa balansae*	*L. infantum* amastigotes at IC_{50} of 0.04 μg/ml	[244]
	Maesabalide III obtained from the leaves of *M. balansae*	*L. donovani* amastigotes at a concentration of 0.8 mg/kg	[245]
	Mimengoside A isolated from the leaves of *Buddleja madagascariensis*	*L. infantum* promastigotes	[246,247]

nanosphere-encapsulated piperine (LN-P), lipid nanosphere-encapsulated piperine with stearylamine (LN-P-SA) and pegylated lipid nanosphere-encapsulated piperine (LN-P-PEG) was injected intravenously to BALB/c mice infected with *L. donovani*. Piperine reduced the liver and spleen parasite load by 38 and 31%, respectively, 15 days p.i., whereas LN-P reduced the parasite burden in liver and spleen by 63 and 52%, respectively, LN-P-PEG reduced the parasite burden in liver and spleen by 78 and 75%, respectively, and LN-P-SA reduced the parasite burden in liver and spleen by 90 and

Table 8. Phenolic derivatives having antileishmanial activity.

Category	Plant metabolites and their source	Active against	Ref.
Chalcones	Licochalcone A obtained from the roots of *Glycyrrhiza* spp.	*L. donovani* promastigotes	[248,249]
	Sulfuretin	*L. donovani* amastigotes at EC_{50} of 1.24 μg/ml and promastigotes at EC_{50} of 0.09-0.11 μg/ml	[250]
	(*E*)-1-[2,4-hydroxy-3-(3-methylbut-2-enyl) phenyl]-3-[4-hydroxy-3-(3-methylbut-2-enyl) phenyl]-prop-2-en-1-one	*L. donovani* promastigotes	[251]
Flavonoids	Amentoflavone, Podocarpusflavone A and B isolated from the leaves of *Celanodendron mexicanum*	*L. donovani* promastigotes	[239]

Table 9. Other metabolites having antileishmanial activity.

Category	Plant metabolites and their source	Active against	Ref.
Acetogenins	Rolliniastatin-1 obtained from the stem bark of *R. emarginata*	*L. donovani* promastigotes	[213]
	Senegalene, Squamocine, Asimicine and Molvizarine isolated from the seeds of *Annona senegalensis*	*L. donovani* promastigotes at concentrations between 25 and 100 μg/ml	[252]
	Annonacin A and Goniothalamicin obtained from the seeds of *Annona glauca*	*L. donovani* promastigotes	[253]
Miscellaneous	Argentilactone obtained from the roots of *Annona haemantantha*	*L. donovani* promastigotes	[254]
	PAP-S and PAP-R obtained from the seeds and roots of *Phytolacca americana*	*L. infantum*	[240]
	Ricin produced by *Ricinus communis*	*L. infantum*	[240]

85%, respectively. Sinha *et al.* [194] also targeted *L. donovani* parasite in the hamster model of VL by encapsulating an antileishmanial compound, andrographolide (a labdane diterpenoid isolated from the Indian medicinal plant *Andrographis paniculata*), in mannosylated liposomes. Mannosylated liposomes were found to be most potent at inhibiting the splenic parasite burden as well as in reducing the hepatic and renal toxicity as compared with free and liposomal forms of compound. Mannosylated liposomes loaded with hamycin have also been reported to show enhanced activity against VL in hamster model. At an equivalent dose of 0.5 mg/kg, administered every 3 days for a total of 3 doses in 7 days, the mannose-coated liposomal hamycin was found to be most effective at reducing splenic parasite load compared with either liposomal hamycin or free hamycin [195]. These delivery systems are summarized in **Table 11**.

8. Conclusions

Despite significant developments in various fields, VL remains a serious public health problem in many parts of the world. Several chemotherapeutic agents have been investigated parenterally as well as orally for the treatment of VL. The classic treatment of VL has, for many years, been pentavalent antimony. Only a few new drugs have been introduced over the years as second-line therapy in case of antimonial failure. One success has been the introduction of miltefosine for VL treatment, although there is still a need for new drugs to provide better therapeutic index and to reduce side effects. The toxic side effects of current antimicrobials and growing resistance of the parasite to the antimonials and pentamidine made the treatment more complicated. Natural plant products have also been investigated widely for their antileishmanial activities. Unfortunately, most of them do not meet all the requirements considered to be essential for their potential commercialization: to be administered topically or orally, to be effective at moderate doses, and not to cause severe side effects.

Advances in the development of DDSs opened up a new era in the treatment of VL. The use of colloidal drug carriers (i.e., liposomes, niosomes, emulsomes, micro/nanoparticles) to deliver more efficiently antileishmanial agents inside the cells is supported by numerous studies. This strategy provides one solution to the problem of the poor penetration and retention of drugs within the phagosomes. The authors have broadly assessed various conventional drugs and DDSs investigated against VL. What can be learnt from these experiments? First, if DDSs are compared with drugs, the various DDSs are found

Table 10. Various extracts prepared from natural plants showing antileishmanial activity.

Plant	Extract/plant part used	Active against	Ref.
S. punctata	Ethanolic extract of leaves	*L. donovani* at IC_{50} of 25 nM	[221]
P. galioides	Ethanolic extract of entire plant	*L. chagasi*	[232]
P. galioides	Petroleum ether extract of entire plant	*L. donovani*	[232]
A. glauca	Dichloromethane extract of seeds	*L. donovani*	[253]
Tinospora sinensis	Ethanolic extract	*L. donovani* amastigotes at IC_{50} of 29.8 ± 3.4 μg/ml and promastigotes at IC_{50} of 37.6 ± 6.2 μg/ml	[255]
Piper betle	Ethanolic extract of leaves	*L. donovani* amastigotes at IC_{50} of 5.45 μg/ml and promastigotes at IC_{50} of 9.8 μg/ml	[256]
Dysoxylum binectariferum	Ethanolic extract of stem bark	*L. donovani* amastigotes at a concentration of 100 μg/ml	[257]
Harungana madagascarensis	Methanolic extract of seeds	*L. donovani* at IC_{50} of < 5 μg/ml	[258]
Aloe vera	DMSO extract of leaf exudates	*L. donovani* promastigotes at IC_{50} of 110 μg/ml	[259]
Artemisia indica	Ethanolic extract of leaves	*L. donovani* promastigotes at IC_{50} of 0.21 mg/ml and *L. infantum* promastigotes at IC_{50} of 0.39 mg/ml	[260]
Desmodium gangeticum	Ethanolic extract	*L. donovani* at a dose of 250 mg/kg	[261]
Triclisia patens	Methanolic and aqueous extracts	*L. donovani* at IC_{50} of 1.5 μg/ml	[262]
Swinglea glutinosa	Methylene chloride extract of bark	*L. infantum* amastigotes and promastigotes	[262]
Protium amplum	Methylene chloride extract of fruits	*L. infantum* amastigotes and promastigotes	[263]
Pelargonium sidoides	Ethanolic extract	*L. donovani* amastigotes at EC_{50} of < 0.1 – 3.3 μg/ml	[264]
P. kurroa	Ethanolic extract of roots and rhizomes	*L. donovani* at a dose of 12.5 mg/kg	[265]
Scrophularia scorodonia	Methanolic extract of flowers	*L. infantum*	[266]
Gongronema latifolia	Methanolic extract of leaves	*L. donovani* promastigotes at a concentration of 50 μg/ml	[267]
Crotalaria barbata	Ethanolic extract of entire plant	*L. donovani*	[268]

Table 11. Various delivery systems encapsulating plant products developed against visceral leishmaniasis.

Carrier systems	Plant product encapsulated	Animal model used	Ref.
Oil-in-water microemulsions and poly(DL-lactide) nanoparticles	Bassic acid	*L. donovani*-infected hamsters	[186]
Liposomes, niosomes and nanoparticles	Harmine	*L. donovani*-infected hamsters	[187]
Nanogels and poly(DL-lactide) nanoparticles	Arjunglucoside I	Hamster model of VL	[188]
Liposomes, niosomes, microspheres and nanoparticles	Quercetin	*L. donovani*-infected hamsters	[189]
Liposomes and niosomes	Amarogentin	*L. donovani*-infected hamsters	[190]
Niosomes, microspheres, nanoparticles and liposomes	Bacopasaponin C	*L. donovani*-infected hamsters	[191]
Mannose-coated liposomes	Piperine	*L. donovani*-infected hamsters	[192]
Oil-in-water emulsions (lipid nanospheres) or fat emulsions	Piperine	*L. donovani*-infected BALB/c mice	[193]
Mannosylated liposomes	Andrographolide	*L. donovani*-infected hamsters	[194]
Mannose-coated liposomes	Hamycin	Hamster model of VL	[195]

to be better than conventional drugs at equivalent concentrations when tested in the parasitic infection, VL. Second, if DDSs are compared in particular, the studies presented show that the drugs encapsulated in liposomes have shown ~ 80% efficacy in the treatment of VL. The number of studies carried out with polymeric particles is rather less than liposomes with ~ 75% efficacy. Niosomes have also shown good efficacy that is comparable to liposomes, but not much work has been done on this carrier system. A little work has also been done on emulsomes and polymeric conjugates, which have also shown good efficacy. The superior efficacy of the encapsulated drug inside the DDSs in eliminating intracellular amastigotes of

leishmania parasite both in the *in vitro* macrophage model and in the *in vivo* animal model of leishmaniasis demonstrates the effectiveness of the drug delivery approach. The mechanism by which these carriers improved the therapeutic index of the conventional drugs in the treatment of VL is presumed to be a facilitated delivery of the drugs to macrophages of the liver and spleen either through natural affinity of these carriers or through macrophage-associated receptors. These DDSs are clearly non-toxic, and because their physicochemical characteristics are different, their pharmacokinetics and comparative effectiveness in various tissues may differ in long-term assays. Although some of these carriers require further evaluation, the results obtained so far are promising.

Drug resistance remains an important obstacle towards better outcomes in treatment by conventional drug therapy. Resistance provided in the case of conventional therapy can also be overcome by the newer approach of these DDSs. Colloidal drug carriers such as liposomes and nanoparticles are able to modify the distribution of an associated drug substance which can overcome the drug resistance. Alternatively, drug delivery approaches using nanocolloidal carriers can in principle target drugs to tissue, cellular and subcellular target sites. By increasing bioavailability of drugs at sites of action, these approaches may provide therapeutic advantages, including enhanced efficacy against resistance. These approaches seek to overcome drug resistance by more efficient delivery to target cells and in some cases by concomitant avoidance or inhibition of drug efflux mechanisms [196].

In general, targeting drugs/natural plant products using the colloidal carrier systems, that is, liposomes, emulsomes, niosomes or micro/nanopolymeric spheres/particles, and so on, to the site of infection could readily be utilized in terms of their industrial application as this can provide a better therapy mode for treatment of VL in comparison with the currently available drug regimen in the market for this disease. High loading efficiency and protracted release profile of these carriers may reduce further the dose size and dose frequency. Further, the easier ligation of surface-specific ligands could enhance the target specificity and performance efficiency of these carriers. Thus, the drugs/natural plant products, which are well known for their effectiveness, however compromised owing to their contraindicated manifestations, can safely be administered for effective cure of VL in the form of carriers. However, the new side effects generated from the use of these DDSs need to be studied. More advances in drug delivery technology will hopefully result in more efficient and less toxic antileishmanial therapeutic regimens.

9. Expert opinion

In the past few decades many advances have been made in the field of delivery systems containing drugs against VL. Various drugs have been the key treatment for VL; but to prevent the drugs' pharmacological and toxicological manifestations before reaching the RES, there is an urgent need to deliver the drugs in the immediate vicinity of the required site. Various DDSs have solved this problem to a great extent. After reaching the bloodstream, they must be rapidly recognized and withdrawn from the circulation by the phagocytic cells of the RES (where the pathogen is located) to achieve elevated drug concentrations in the target cells. They should also allow sustained release of the drug, to attain therapeutic levels at the site over prolonged periods of time. They must be able to increase the therapeutic index of the drug, decreasing its toxicity and maintaining its therapeutic efficacy. Various DDSs, such as liposomes, emulsomes, and so on, offer distinctive advantages, such as high loading and the prospect of controlling size and permeability, thus controlling the release kinetics of the drugs from the carrier system. Uptake of the carrier system by macrophages increases appreciably when ligands capable of interacting specifically with surface receptors of macrophages are incorporated. In addition, macrophage-specific targeting could eradicate intracellular parasites by increasing the localized manifold concentration of the drug, and by reducing contraindicated manifestations resulting from systemic drug effects [115,116]. Thus, the site-specific targeting could offer a multitude of clinically viable strategies, which may become a great tool in the treatment and management of VL.

Among all the DDSs discussed, liposomes are the most extensively studied carrier system with drugs as well as plant products and AmB is the most extensively tested drug with almost all the carrier systems studied. AmB has shown significant efficacy with all the carrier systems compared with conventional drug. Likewise, data obtained so far for AmB emulsomes are encouraging. AmB liposomes are already on the market but owing to long-term stability problem of liposomes there is an emerging need of some other delivery system that can be stable as well as significantly effective. In this sense AmB emulsomes seem to be the most promising alternative of the liposomes against VL. Moreover, contrary to pentavalent antimonials and pentamidine, the problem of resistance is not encountered with AmB therapy. Although incorporation of AmB in other carriers reduces its toxicity and increases the therapeutic index for intravenous administration, the lipophilic nature of the drug AmB makes it the best candidate to load inside the internal oily core of the emulsomes. Similarly, because of the nanometric size of emulsomes they can also be administered intravenously and intracardially and can be taken up by the reticuloendothelial system (target site for VL), similar to liposomes. The composition and manufacturing procedures of the emulsomes make feasible the production of a stable final product that could be an economically interesting alternative to the liposomal formulation available at present [115,116].

Nanotechnology can provide the means and materials to build DDSs in the nano size range. Being in the nano size range, these delivery systems can be administered parenterally and taken up preferably by the macrophages [126]. AmBisome, the first nanomedicine available on market for the treatment

of VL, has shown effectiveness with reduced toxicity, shorter treatment period and response with a single dose; but owing to its unaffordable cost ($20 per 50 mg vial) this treatment is available only to a small fraction of the diseased population. Similarly, the prices of most VL drugs are high, which in turn increases the cost of DDSs, therefore WHO should continue to negotiate with manufacturers, as they have done successfully recently. Generic production should be promoted to increase competition and remedy the vulnerable situation in which VL drugs and DDSs are produced by only one manufacturer. To guard against unregulated use, the cash value of antileishmanial drugs and DDSs needs to be minimized by the provision of free treatment to patients. Prevention of resistance also needs to include monitoring of the effectiveness of treatment, relapse rates and cure rates of relapsed patients, and the establishment of a method for *in vitro* drug susceptibility testing [63].

A common practice can be to use glycoproteins or polysaccharides ending in mannose or fucose radicals, and polyanionic macromolecules such as acetylated LDL lipoproteins, having affinity for macrophage receptors [117]. Another alternative is the fixation of specific antibodies of the agent responsible for the infection, such that the selectivity for infected cells is increased. Special attention should be paid to biological carrier systems such as cell ghosts, which are highly biocompatible and, owing to their specific delivery to phagocytic cells, seem to be promising DDSs for the treatment of VL.

The recognition of healthcare resource limitation has produced awareness that new DDSs must be not only highly efficacious, but also cost-effective. DDSs that treat VL more efficiently and possess fewer side effects will have clear and definite advantages. However, these advantages must be achieved at reasonable and competitive prices in terms of alternative treatments, decreased hospitalization time, and/or more rapid patient recovery for return to active work. Owing to their complexity, site-directed DDSs such as ligand-directed carriers undoubtedly will cost more to develop and manufacture than conventional therapeutic agents. The resulting higher prices may be acceptable if cost increases can be minimized and if performance of these DDSs is increased sufficiently to produce overall savings to the healthcare system [197]. These are significant issues that must be considered thoroughly when designing the delivery system because they ultimately will determine the product's success in the clinical marketplace.

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Declaration of interest

The authors state no conflicts of interest.

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