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Review Article

MACROPHAGE TARGETING: A STRATEGY FOR LEISHMANIASIS SPECIFIC DELIVERY

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ABSTRACT

Leishmaniasis is a vector-borne zoonotic infection caused by an obligate intra macrophage protozoan parasite '*Leishmania*'. Despite of a number of remedies available, leishmaniasis is still a speedy migrating and deadly infection due to the resistance of the parasite to the drugs as well as their toxicity. Hence, there is a need for targeted drug delivery system for enhancing the systematic effect of antileishmanial drugs. Although the number of antileishmanial drugs in a variety of dosage forms is available, there is an urgency to develop more efficient, cost-effective and safe therapy, which can be achieved by macrophage targeting utilizing passive (phagocytosis), and/or active (receptor mediated) strategies utilizing nano-formulations. Positive considerations of various factors like the enhanced permeation and retention (EPR) effect, size, and charge of nano-formulations can facilitate the passive targeting, and various receptors like lectin receptor, mannose receptor, mannosyl-fucosyl receptor, scavenger receptor, etc on the macrophage surface may play an important role in active drug targeting. Also, monoclonal antibody, interferon's, tufstin are other agents which have been broadly utilized for targeting.

Keywords: Leishmaniasis, Macrophages, Targeting, Receptor, Nano-formulations

INTRODUCTION

This review provides a current perspective on the challenges and possibilities in the macrophage targeting. It summarizes recent important and interesting articles investigating the challenging treatment of the parasitic infection, leishmaniasis. In addition, it compares and contrasts targeting strategies for leishmaniasis. We retrospectively reviewed all articles from 1950 to 2015 focused on Leishmaniasis, by searching in the Pub Med database, Science direct, wiley online library on each of the following keywords: '*Leishmania*', 'macrophage targeting', 'drug delivery systems', 'drugs 'life cycle', 'conventional therapy', 'kala-azar', 'receptor', 'nano-formulations', in association with 'leishmaniasis'. After vast literature survey and selection procedure, this article comes into form discussing the issues, and future possibilities in the treatment of leishmaniasis Leishmaniasis represents an assembly of diseases with clinical and epidemiological diversities. It encompasses a range of clinical manifestations that initiate from simple self-limiting, self-healing or asymptomatic cutaneous ulcers and may proceed to horribly disfigure, debilitating mucocutaneous and lethal visceral form if remains untreated [1]. Leishmaniasis is a vector born zoonotic infection caused by obligate intra macrophage protozoan parasite '*leishmania*' in the mammalian hosts and spreads by carrier female sand fly. Despite of a number of remedies available, leishmaniasis is still a speedy migrating deadly infection, which is broadly attributed to the resistance of the parasite to drugs as well as their toxicity. Besides, intersection with human immunodeficiency virus (HIV) with a predictable increase in treatment failures, and large-scale resistance to antimonial has generated an urgency to develop more efficient panacea. Further, in this context chemotherapy has been hampered by localization of parasites within lysosomal vacuoles of the macrophages, restricting the bioavailability of many potential antileishmanial compounds.

Lacuna in the treatment regimen provokes to generate a therapy that is cheaper, requires a minimal dose of drugs and specific to its site of action. Since, the causative agent of the disease, an intracellular parasite harbour in macrophages and specific tissue sites, which can be the target of the drug, the targeted drug delivery system is the need of the day [2].

Various *leishmania* species infect macrophages and dendritic cells of the host immune system, causing symptom ranging from disfiguring cutaneous and mucocutaneous lesions, widespread destruction of mucous membranes, or visceral disease affecting the haemopoetic organs of the host. This infection can be broadly classified into three clinical syndromes namely cutaneous leishmaniasis (oriental sore), mucocutaneous leishmaniasis (espundia) and visceral leishmaniasis (VL, kala-azar). Other forms are posted kala-azar dermal leishmaniasis, viscerotropic leishmaniasis and diffuse cutaneous leishmaniasis. Clinical manifestation of the disease depends on the species (*Leishmania donovani, L. infantum* or *L. major*) involved.

Host–parasite interaction: life cycle of *leishmania* parasite

Stage 1: Development of amastigote in sandfly

L. donovoni, the causative agent of Leishmaniasis, is an obligate parasite which is carried by the bloodsucker adult female sandfly from the infected mammalian host. Inside the midgut of the sandfly, the amastigote form of this parasite multiplies within 4-25 d via binary fission and finally transforms into promastigote. Promastigote live extracellularly in the alimentary canal of the sandfly, reproduces asexually and migrates to the proximal end of the gut for transmission to the mammalian host. The life cycle of *L. donovani* is represented schematically in fig. 1.

Stage 2: Development of promastigote in mammalian host

Host invasion involves attachment to macrophages, phagocytosis, and development inside the phagolysosome. Promastigotes enters the mammalian host along with the sandfly's saliva out of which some are destroyed, and the others are phagocytosed by the macrophages. The parasite resides intracellularly in the parasitophorous vacuoles of the macrophages, an acid and lytic compartment presenting the hostile ecological niche. The host macrophage phagocytes the promastigotes via receptor-mediated endocytosis where phagosome forms phagolysosome, after fusion with the lysosomes. Various macrophage receptors involved in the entry of promastigotes are the mannose-fucose receptors, receptors for advanced glycosylation end products (AGE), fibronectin receptors, Fc receptors (FcR) and the complement receptors CR1 and CR3 [3]. During the later phase, promastigotes (the extracellular form) differentiate into amastigotes (the intracellular form) thus adapting to live in the hydrolytic environment of the lysosome. It has been proposed that *L. donovani* after a certain extent of the reproduction, lyse their host cell owing to the sheer pressure of the mass, but few speculations also claim that they are able to leave via exocytosis. *In vivo* studies indicate that once inside macrophages,

promastigotes start to differentiate into amastigotes only when exposed to the acidic environment, and this occurs after infected phagosomes fuse with late endosomes. These amastigotes invade the other macrophages, utilizing various mechanisms of immune evasion like down-regulation of the parasite antigen presentation via major histocompatibility complex (MHC) Class II pathway, inhibition of oxidative burst, etc. This parasite causes the systemic infection of the entire reticuloendothelial system (RES), liver and spleen. Some of the free amastigotes are taken up by the sandfly in their blood meal and thus the cycle continues [4-7]. The parasite is presented to the T cells in draining lymph nodes via dendritic cells from the infected site [8]. Multiplication of the amastigotes in the macrophages takes place until the release occurs by a burst of macrophages.

Cons with the current therapies being practiced in management of leishmaniasis

Leishmaniasis has been mainly controlled by chemotherapy. Pentavalent antimonials exploited since the last 6-7 decades for the treatment of leishmaniasis have long been the mainline treatment, but, are now not very effective due to protozoal resistance. Moreover, the side effects of currently available antimicrobials and increasing cases of resistance of *Leishmania* to the antimonials and pentamidine have turned the situation to be more complicated. Over the past decade, although alternative drugs or new formulations of other standard drugs have become available and some other drugs are under clinical trials, the problem is still critical, and an effective approach is yet to be sorted out.

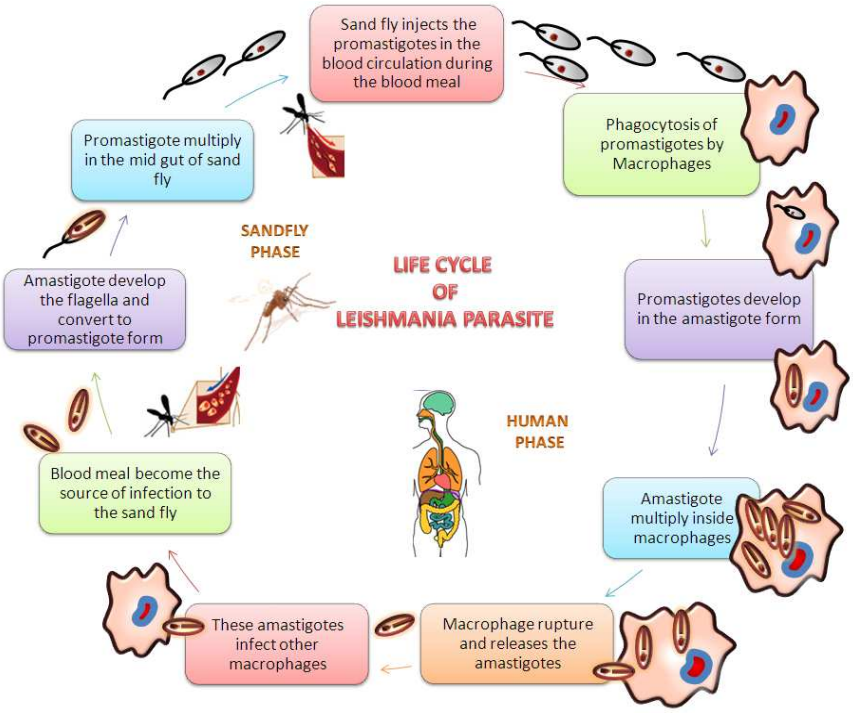

Fig. 1: Schematic representation of life cycle of Leishmania

Recently, a success has been the introduction of Paromomycin (an aminoglycoside antibiotic) but still there is the need to provide a better therapeutic index and to reduce side effects. Furthermore, the aim to develop a single drug or formulation drug effective against all forms of leishmaniasis still remains a dream. In the present scenario, the majority of the conventional dosage forms, following 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 [9]. The high drug clearance from the body is also a limitation of conventional drugs. Besides, the current treatment regimen requires a prolonged course and treatment is often conducted in a hospital set up. table 1 provides an insight to the conventional therapy regimen and also lists the potential toxic effects of antileishmanial drugs.

The failure of existing conventional therapy may in part be attributed to lack of *in vitro-in vivo* co-relation. Therapy failure arises as the drugs which show excellent *in vitro* data are often followed by poor *in vivo* results. Unpredictable bioavailability, rapid first pass metabolism, augmented clearance are some reasons that may be cited as the cause of drug failure.

Other useful drugs against leishmaniasis include Amphotericin B (AmB) and Miltefosine. A recent survey in Bihar (most affected place in India, more than 90% alone) had recorded an alarming 1,000,000 cases with 10,000 unresponsive to Antimonials, Pentamidine, and Am B [10-12]. Thus, it becomes obvious to develop targeting strategies instrumental in providing spatial delivery of more effective, less resistant anti-leishmanial drugs with an interception of minimal side effects.

Macrophages targeting

Designing a system proficient in delivering selectively the drug to the site of action is the main aspect of drug targeting. Macrophage-targeted drug delivery approach may serve as a promising means to conquer many of the aforesaid problems. Localization of leishmania parasite within the phagolysosome of macrophages restricts the bioavailability of many potentially useful antileishmanial drugs. Macrophages serve as host cells for these parasites which are able to inhibit phagosomes maturation so as to survive and replicate within the macrophages. Hence, it is difficult enough to provide access of drugs selectively to these relatively inaccessible sites. As a result, macrophage-specific drug delivery systems are the focus of interest. Therefore, it becomes imperative to exploit the concept of “magic bullet”, a novel concept of drug targeting paradigm that exploits the use of surface engineered vehicles for site specific delivery. Further, a targeting system needs to be designed keeping in view the ultimate site of action of drug which in the present case are macrophages. Thus, in the scenario, the carrier should be so conditioned that they are directed to buffer's cells either passively or otherwise actively.

Table 1: Conventional therapy for leishmaniasis

Drug → Parameter ↓	Sodium stibogluconate	Meglumine antimoniate	Pentamidine	Amphotericin B	Paromomycin	Miltefosine	Stigmaquine
Chemical nature	Antimonial compound	Antimonial compound	Aromatic Diamidine	Polyene	Aminoglycoside	Alkylphospholipid	8-aminoquinoline
Dose	20 mg/kg/day for 30 d	20 mg/kg/day for 20-28 d	2-4 mg/kg for 15 d	1-3 mg/kg for 20 d	15 mg/kg/day for 21 d	2.5 mg/kg/day for 28 d	1.75-2.5 mg/kg for 28 d
Route of administration	iv, im	iv, im	iv, im	Iv	Im	Oral	im
Side effects	Cardiotoxicity, pancreatitis, renal tubular dysfunction and musculoskeletal pain	Fever, irregular heartbeat, nausea, pain in the upper abdominal area and vomiting	Insulin dependent diabetes mellitus	Nephrotoxic, cardiac arrhythmia and hypokalemia	Nephrotoxic and eighth cranial nerve toxicity	Teratogenic, hepatotoxic, vomiting and diarrhea	Nephrotoxic
Current status	Marketed, Pentostam (Resistant)	Marketed, Glucantime	Marketed	Marketed, Fungizone	Phase III clinical trials	Phase IV clinical trials	Phase II clinical trials
References	[13-16]	[11, 17]	[13, 15, 18]	[13]	[19-21]	[13, 15]	[15, 20, 21]

In spite of the potential benefits of targeted nano-carriers, these systems have some drawbacks such as the cost and stability of the targeting moiety. To justify the increased cost, the moiety must significantly increase the therapeutic efficacy of the nano vector. Transport systems can be designed to control the dispatch of the loaded drug to target areas, increasing its local concentration and bioavailability, while prolonging its retention, half-life, and effectiveness.

Outstanding favors which can be obtained by this method are: improved pharmacokinetics, independence of the administration method, minimization of required amount of the drug and its side effect and hence the cost of the therapy. Such researched novel drug delivery systems loaded with anti-leishmanial drugs are cited in table 2. One further drawback of targeted delivery is the effect the uptake pathway may have on these systems. The targeting moiety has to be specific to the area of interest.

Table 2: Drug delivery systems studied for the delivery of antileishmanial drugs

Drug delivery system	Drug encapsulated	Parasite studied	References
Liposomes	Antimonial	*L. Donovani, L. Major*	[41]
	Camptothecin	*L Donovani*	[34]
	Pentamidine	*L. Donovani*	[42]
	Miltefosine	*L. Donovani*	[45]
	Atovaquone	*L. Donovani*	[46]
Niosomes	Amphotericin B	*L. Donovani*	[47]
	Sodium stibogluconate	*L. Donovani*	[48-51]
Microparticle	Sodium stibogluconate	*L. Donovani*	[52]
	Amphotericin B	*L. Infantam*	[53,54]
	Doxorubicin		[55]
Nanoparticle	Amphotericin B	*L. Donovani (In-vitro)*	[38, 56]
	Pentamidine	*L. Infantam*	[57]
	Pentamidine	*L. Infantam*	[57, 58]
	Primaquine	*L. Donovani*	
	Primaquine	*L. Donovani*	[24]
Nanosuspension	Amphotericin B	*L. Donovani* (oral)	[59]
	Aphidicolin	*L. Donovani (In-vitro)*	[60]
Solid lipid nanoparticles	Amphotericin B	*L. Donovani*	[61]
Emulsion	Amphotericin B	*L. Donovani*	[62-65]
	Amphotericin B	*L. Donovani*	[62-65]
	Piperine	*L. Donovani*	[43]
	Sodium stibogluconate	*L. Donovani*	[66]
	Sitamaquine	*L. Major* (topical)	[67]
Drug conjugate	Sodium stibogluconate	*L. Donovani*	[68]
	Amphotericin B	*L. Major*	[69]
	8-aminoquinoline	*L. Donovani*	[70]

Concept of passive targeting

Immunological response to foreign moiety is the natural phenomenon of the body which can be utilized for the passive targeting mediated by the invasion of the drug and drug carrier systems on the basis of their physicochemical properties. Particles uptake by the cells of the RES is an excellent example of the passive targeting. The endeavor potential of macrophages for rapid recognition and clearance of foreign particles has provided a rational approach to macrophage-specific targeting with nano-carriers. Passive capture of colloidal carriers by macrophages offers therapeutic opportunities for delivery of antileishmanial drugs in leishmaniasis since it involves macrophages cells of the RES (fig. 2).

Phagocytosis: a mechanism of passive targeting

Phagocytosis 'a biological phenomenon' is carried out by specialized cells of the mononuclear phagocytic system (MPS) called

phagocytes. Phagocytosis is mediated by the adsorption of specific blood components (e. g. immunoglobulin IgG, complement C3b, and fibronectin) called opsonins and binding of pertinent receptors located on macrophages to the entity being phagocytosed. As the ingestion of moiety takes place, the phagocytic vacuole (or phagosome) fuses with one or more lysosomes to form mature phagolysosome (secondary lysosomes), and this occurs through a succession of transient fusion proceedings with early endosomes (EE), late endosomes (LE) and lysosomes (Ly). This maturation progression allows phagosomes to acquire some of their microbicidal properties and the ability to process antigens. All the way through evolution, intracellular pathogens have developed diverse strategies to avoid killing in phagolysosome [22]. The promastigote form of *Leishmania* alters phagosome maturation by inhibiting fusion with late endosomes and lysosomes. This allows these pathogens to reside in phagosomes displaying early endosome-like features that are not able to kill and degrade microorganisms. On the other hand, amastigote form of *Leishmania* seems to be able to survive in the harsh environment of phagolysosome. Thus uptake of drug loaded carriers by the RES system followed by digestion of carrier by lysosomal acid hydrolysase, subsequently releasing the drug may work in co-ordination. This would allow the drug to exert selectively its therapeutic effect and thus eliminate the parasite. Primaquine, when loaded in polyisohexylcyano-acrylate [23] and poly alkyl cyanoacrylate [24] nano particles showed superior antileishmanial activity as compared with free drug. The drug-loaded polyisohexyl cyanoacrylate nano particles showed a 21-fold increase in antileishmanial activity as compared with the free drug when evaluated *in vitro* using J774G8 macrophage-like cells infected with *L. donovani*. Primaquine loaded poly (DL-lactide) nano particles were found to be 3.3 times more effective than that of the free drug in terms of amastigote suppression in the liver [25]. Moreover, these nano particles were found to be non-toxic [26]. Another study reports the potential application of biodegradable polymer methoxy poly-(ethylene glycol)-b-poly (lactic acid) nano particles MPEG-PLA NPs loaded with doxorubicin and mitomycin C as a method for targeted drug delivery to macrophages with fewer side effects [27].

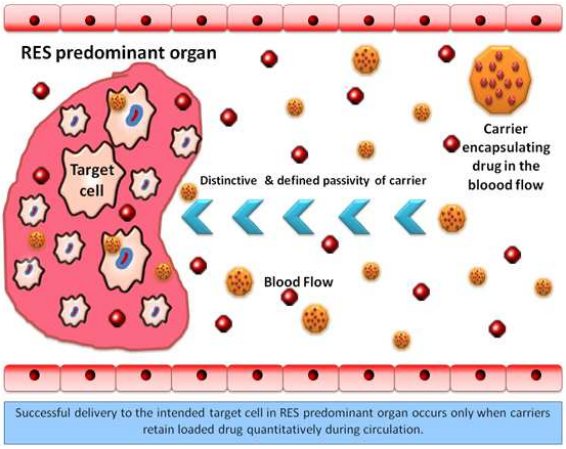

Fig. 2: Schematic representation of passive targeting to leishmaniasis infected macrophages of RES

Factors facilitating passive targeting

In the past few decades, many advances have been made in the field of delivery systems containing drugs against *Leishmania* parasite. This advancement in technology rekindles hope for treatment of the disease. Various drugs have been the key treatment for leishmaniasis, but to prevent the pharmacological and toxicological mani-festations of the drug before reaching the RES, there exists an urgent need to deliver the drugs in the immediate vicinity of the required site. The passive target ability attributed to 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 disease cell lines [28]. Passive targeting is being exaggerated by pathophysiological factors (inflammation/infection and enhanced permeation and retention effect) as well as physicochemical factors (size, surface charge, and molecular weight) of the drug delivery system.

Inflammation/infection and enhanced permeation and retention (EPR)

Macrophages are regulators of inflammation in many infectious diseases as they secrete a multitude of inflammatory mediators and hence serve as the potential pharmaceutical target for various animals and human diseases. Although, some of the microorganisms like Toxoplasma gondii, Leishmania sp, Mycobacterium tuberculosis, and Listeria monocytogenes have developed the potential ability to resist this phagocytosis activity, carrier-mediated delivery of antimicrobial agent (s) into pathogen-containing intracellular vacuoles in macrophages could be useful to eliminate these cellular reservoirs [29-30]. This could reduce side effects associated with the drug administration and also release of pro-inflammatory cytokines and at the same time will help to achieve therapeutic drug concentrations in the vacuoles of infected macrophages. Hence, nano-systems designed utilizing above approach may be immensely useful in macrophage targeting of disease like leishmaniasis (table 2).

Increase in permeability of blood vessels due to infectious diseases results in the leaky vasculature, allows migration of 10-500 nm diametric particles across the blood vessel walls and intestinal space [31]. The nature of the disease affects the porosity of the vasculature, allowing for control over the diffusion of the drug; the choice of a properly sized carrier would allow the drug to extravasate from the blood vessel. This phenomenon is termed as “Enhanced Permeation and Retention (EPR) effect” [32]. Drug carriers should, however, circulate in the blood long enough to provide acceptable accumulation of the active molecule in the area of interest. To overcome this dilemma 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 ‘stealth’ effect makes them long circulatory in nature. The adsorption of Silicone-glycol copolymers on the surface of liposomes was utilized for their steric stabilization [33]. These sterically stabilized vesicles showed enhanced half-life. In a similar study, sterically stabilized polyethylene glycol (PEG) coupled liposomes were developed and studied for the antileishmanial efficacy of camptothecin (CPT) in the free and liposomal form *in vitro* against L. *donovani* promastigote as well as *in vivo* in a murine model of VL. Treatment of infected mice intraperitoneally with free and liposomal CPT significantly reduced the hepatic parasite loads by 43 and 55%, respectively as compared with loads of untreated controls [34].

Size

The depth of penetration in the target tissue has always been a problem with targeted drug delivery. It has been illustrated that after extravasation from the vasculature, the targeted carriers bind to the first few cell layers in retarding the entry of following carriers [35]. This phenomenon is correlated with the size of the nano vector and binding affinity of the targeting ligand. The bigger nanovectors and stronger binding ligands penetrate shorter distances. 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 bio distribution of systemically administered drugs. The effectiveness of drug delivery systems can be manipulated by an alteration in their size, which can facilitate controlled release of the drug, modification of drug pharmacokinetics and biological distribution and also reduced drug toxicity.

When novel liposomal formulation of meglumine anti-moniate consisting of reduced size vesicles was evaluated in dogs having VL, it was found that passive targeting of liposomes to the bone marrow of infected host was improved by the reduction in vesicular size to the nanoscale. In this study, the pharmacokinetics of antimonial drug was assessed in the blood and in organs of the mononuclear

phagocytic system for the liposomes of vesicular size 400 nm and compared with free drug and drug encapsulated large sized liposomes (mean diameter 1200 nm). The reduction in vesicle size from micrometer to nano size range exhibited direct passive targeting of liposomes to the bone marrow of dogs infected with VL [36]. Hence, the size of 150-250 nm may be considered appropriate to be actively phagocytosed by macrophages. Apart from size, a surface characteristic of the moiety also decides the degree of opsonization. The Greater hydrophilic surface of the system leads to decreased opsonization and phagocytic uptake. Miltefosine (MF) loaded albumin microparticles of size range 2-5 µm were prepared by spray drying method combined with thermal stabilizer. The formulation was found suitable to target the macrophages as 65% of RAW macrophages cells engulf MF microparticles within 90 min when administered parenterally. The microparticles also displayed less hemolytic toxicity as compared to free MF [37].

In a study by Nahar *et al.*, PLGA nanoparticles (PNPs) encapsulating AmB were prepared by emulsion solvent evaporation method. The developed system was found to be in nanometric size (168 nm) and showed 85% inhibition against promastigote model and the activity of AmB as a plain drug, Ambisome and PNPs against *L Donovani* in intra-amastigote macrophage model showed 71.77%, 83.03% and 84.06% inhibition respectively [38]. In another study Singh *et al.,* evaluated the antileishmanial efficacy of Am B bearing polycaprolactone (PCL) microparticles. The microparticles were prepared, optimized and subjected to *in vitro* characterization for shape (spherically structured), particle size (9.83±1.12 micron), entrapment efficiency (43.54±3.98%) and *in vitro* drug release and revealed their efficacy against leishmanial parasites residing in macrophages [39].

Surface charge

The charged phospholipids contained in liposomes greatly enhance their binding to the macrophages, which in turn are consequently engulfed by these cells. This offered a strategy for targeting VL based on the development of cationic liposomes composed of positively charged stearylamine-egg phosphatidylcholine-bearing drug liposomes (SA-PC liposomes). Both promastigote and intracellular amastigotes *in vitro* and *in vivo* were susceptible to SA-PC liposomes. A single dose of 55 mg of SA-PC liposomes/animal was able to significantly reduce the hepatic parasite burden by 85 and 68% against recent and established experimental VL, respectively, suggesting their strong therapeutic potential [40].

When drugs are administered in free form in the body, only a small fraction can reach the macrophages, and the remaining fraction of the drug may lead to toxic side effects. This serious problem has generated the need to develop strategies for selective and targeted delivery of drugs to the macrophages. Uptake of the carrier system by macrophages increases appreciably when a charge capable of interacting specifically with the surface of macrophages is incorporated. In a study by Alving *et al.,* (1978), the research group found out that the efficacy of treatment was influenced by the lipid composition and charge of the liposomes. They observed that positively charged liposomes containing egg phosphatidylcholine were much less effective than negatively charged ones, whereas positively and negatively charged sphingomyelin liposomes were found equally effective. Liposomes containing phosphatidylserine (which were negatively charged, but also had a much higher charge density) were among the less-effective preparations. Furthermore, among the tested, liposomal formulations, most consistently efficacious liposomes contained highly saturated long-chain phospholipids (eg. dipalmitoyl phosphatidylcholine), cholesterol, and a negative charge [41].

Concept of active targeting

Active targeting redefines the biofate or natural distribution pattern of the drug carrier system with modification or manipulation of the surface of carriers so that it can be identified by specific cells. Binding of drug loaded carriers to target cells is facilitated by the use of ligands or engineered homing devices and thus enhances the receptor-mediated localization of drug. *Leishmania* parasite resides in intracellular phagolysosome and hence there is a need to target the drug delivery system intracellularly. Intracellular targeting is third order targeting and involves receptor-based ligand-mediated entry of a drug complex into a cell by endocytosis followed by lysosomal degradation of carrier leading to the release of drug.

The uptake of *leishmania* promastigote by macrophages is a receptor-mediated process that involves the expenditure of energy by the macrophage, but not by the parasite. Due to the obligate intracellular nature of the pathogen, this organism expresses several different ligands on its surface that can interact with a variety of different macrophage receptors, to ensure its uptake by phagocytic cells. These include the receptors for complement, fibronectin, sugars (such as the mannose-fucose, galactosyl receptor) and others. Different receptors bind carrier molecules with different avidity. In a study by Banerjee *et al.* (1996), the mannose-grafted pentamidine isothionate liposomes were found to be the most effective, with 85.1% reduction in splenic parasite load as compared with glucose-grafted liposomes (65.9% reduction), galactose-grafted liposomes (45.1% reduction), uncoated liposomes (46.6% reduction) and free drug (18.5% reduction in splenic parasite load) when tested in L. *donovani* infected hamsters [42].

The intracellular localization of the pathogens in the diseases of microbial etiology such as VL necessitates the administration of relatively high doses of cytotoxic drugs 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 [43]. Many approaches for targeting drugs to the macrophages have been developed. Although many carriers show a natural affinity towards the macrophages and are passively targeted to them, the inclusion of the macrophage receptor(s) specific ligands may significantly enhance the rate and extent of their uptake by the macrophages.

These receptors are able to bind to modified lipoproteins, senescent and apoptotic cells, proteins, polysaccharides and a range of poly anionic molecules and control the activities such as activation, recognition and endocytosis [44].

Receptor mediated targeting strategy

The surface of promastigote has phosphoglycans (lipo-phospho-glycans (LPG) and proteophosphoglycans (PPG) and gp63 molecules that serve as ligands for their attachment and subsequent entry in the macrophages [71]. CR1 and CR3 receptors are complementary receptors which facilitate phagocytosis of the promastigote and improve the survival of the parasite via preventing respiratory burst [72-73]. CR4, fibronactin receptors, mannose receptors and advanced glycosylation end product receptors are the other receptors engaged in phagocytosis. In case of amastigote, proteophosphoglycans are involved in the phagocytosis since lipophosphoglycan are absent [74]. CR3, Fc and mannose receptors are receptors on macrophages specifically for uptake of amastigote.

Receptors present on the cell surface of macrophages are complex trans-membrane proteins, which mediate highly specific interactions between cells and their extracellular region. Receptors have highly specialized recognition sites with rigid structural requirements for binding signalling ligand. Exploiting a variety of macrophage cell receptors as therapeutic targets may prove to be a fascinating strategy for delivery and targeting of drugs with particulate nanocarriers [75].

Ligands are carrier associated surface group(s) which can selectively direct the carrier to the pre-specified site(s) housing appropriate receptor units and thereby serveing as 'homing device' of the carrier/drug. The interaction occurs due to their respective receptors localized on the cellular surface as showed in fig. 3. Further, the cellular machinery that drives receptor-mediated events is endocytosis, receptor-mediated endocytosis, clathrin linked endocytosis, ligand-mediated transcytosis, etc.

The potential targeting moieties exploited for selective drug targeting may be widely classified into endogenous ligands, immunological ligands, glycol-conjugates and antibody conjugates. These include antibodies and their fragments, aptamer (protein

binding DNA), peptides, proteins, saccharides, hormones, glycoproteins, fusogenic agents and vitamins, especially folate. However, major concern is that the targeting ligand itself could elicit an immunogenic response in a patient although this issue is more prominent for antibodies.

In a study by Kansal *et al.*, Phosphatidylserine (PS) was used as ligand to target *Leishmania*-infected macrophages by developing Nano capsules (NCs) being doxorubicin (DOX). And, results opened the insight for efficient drug delivery, as PS-NCs-DOX, causing 85.23%±4.49% inhibition of splenic parasitic burden whereas, NCs-DOX and free doxorubicin caused only 72.88%±3.87% and 42.85%±2.11% parasite inhibition, respectively, in Leishmania-infected hamsters [76].

Lectin receptors

Lectins belong to a class of proteins characterized by the ability to bind carbohydrates with high specificity. Design and development of potential carriers for cell specific delivery of therapeutics are immensely dependent on the selectivity of the carrier to the cellular receptors distributed variably at intracellular sites and on the surface of cellular systems. Lectin receptors present on macrophages constitute potential recognition sites for carbohydrate-mediated interaction between the cells and drug carrier bearing suitable site-directing molecules. Banerjee *et al.*, 1996 formulated different sugar-grafted liposomes encapsulating pentamidine isothionate and their methoxy derivative tested *in-vivo* against reversible VL in hamsters. Sugar-grafted liposomes are encapsulating both the drugs were found to be more potent in comparison to plain liposomes encapsulating drug and the free drug [42].

Mannose receptors

Macrophages express mannose-specific endocytotic lectin receptors that bind and internalize mannose-conjugated bio conjugates. The expression of this receptor is tightly modulated during monocyte/Mf differentiation and cellular activation. Receptor activity is dependent on the number of receptors present on the cell surface, the affinity of these receptors for the ligand, the rate of receptor internalization and recycling. Mitra *et al.* compared the antileishmanial property of a benzyl derivative of an antibiotic MT81 (Bz2MT81) in free, liposome-intercalated and mannose grafted liposome-intercalated forms in *L. donovani* infected hamsters [77]. Various formulations were administered subcutaneously at a dose equivalent to 7.5 mg/kg (body weight) for 15 d at an interval of 3 d. In case of mannose grafted liposomes, the splenic parasitic inhibition was 79.1%. Further, free and liposomal drug forms were less effective in reducing the parasite load in spleen (49.8 and 55.1% parasite suppression, respectively). In a similar study, Veera reddy *et al.* developed uncoated and mannose-coated lipid nanospheres of AmB. These formulations were administered to *L. donovani*-infected BALB/c mice at a dose of 5 mg/kg [61]. The same dose of Fungizone solution was also administered to separate mice as control groups. In liver and spleen, the mannose-anchored AmB lipid nanospheres reduced the parasitic burden by 95% and 94%, AmB lipid nanospheres reduced 90% and 85% and Fungizone reduced 82% and 69%, respectively. The tissue distribution studies suggested mannosylated nanospheres to distribute specifically more rapidly to liver and spleen.

Thus, the mannosylated formulation can be deemed to have convincing prospectives in delivering antileishmanial drugs to parasite-infected macrophages. Selective delivery of liposomes may be performed by the mannose receptors on the surface of macrophages [77-80]. Fiani *et al.* (1998) performed ligand binding studies on J774 clone cells. The research group concluded that these cells differ primarily in their levels of mannose receptors on the cell surface, and there may be two levels of regulation. One in which receptor numbers being modulated by receptor synthesis and receptor degradation and the other in which the intracellular itinerary of the receptor is modulated [81].

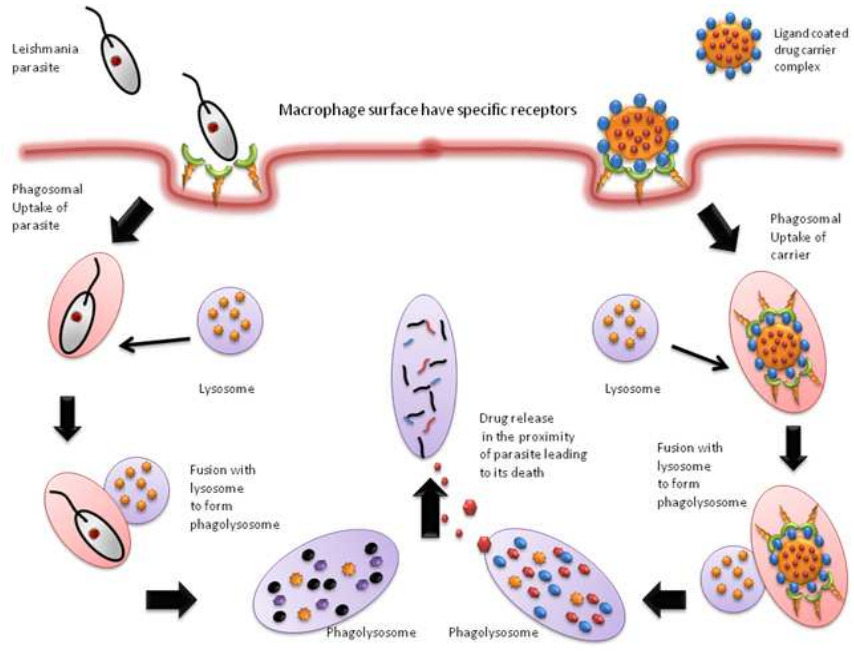

Fig. 3: Schematic representation of active targeting to *Leishmania*-infected macrophages

The development of amastigotes occurs in the phagolysosome of macrophages, and the effective targeting may be performed via the mannose lectin since it is localized at the surface of the macrophages. This may allow specific internalization of mannosylated ligands which are quickly transferred from endocytic vesicles to early endosomes (where receptor-ligand dissociation occurs) and then to the phagolysosome. The active targeting of CpG-containing oligo deoxynucleotide (CpG-ODN) to macrophages was studied by incorporating it in mannose coated liposomes and injecting it in animals with VL as the model macrophage disease. A complete elimination of spleen parasite burden was achieved by mannosylated liposomal CpG-ODN in comparison to 62% and 81% parasite suppression by free and liposomal ODN formulations respectively in 60 d mouse model [82]. Kole *et al.* found that

mannosylated liposomes of doxorubicin were more effective than plain liposomes or free doxorubicin in the treatment of VL in *L. donovani*-infected BALB/c mice [83].

Emulsomes are nano emulsion pharmaceutical compositions comprising of a lipid core that is a solid or liquid crystalline phase and stabilized by at least one phospholipid envelope. These have been found suitable for the parenteral, oral, rectal, intranasal, or topical delivery of both fat-soluble and water-soluble drugs [84, 85]. Trilaurin based nanosized lipid particles (emulsomes) of Amb had been formulated and stabilized by soya phosphatidylcholine as a new intravenous drug delivery system for macrophage targeting. The system was modified via coating with the macrophage-specific targeting ligand O-Palmitoyl mannan (OPM). These OPM grafted emulsomes showed higher *In-vitro* efficacy in comparison to plain AmB emulsomes and AmB-Doc against L. *donovani* infected macrophages amastigotes system. At the same time OPM grafted emulsomes showed more efficient parasitic inhibition (PI), vis a vis 73.7±6.7% in comparison to plain emulsomes (51.7±5.4%) and AmB-Doc (30.4±4.8%) when administered intracardially at alternate days to infected hamsters [65].

Mannosyl–fucosyl receptor

A leishmanicidal drug, HPP-Rib encapsulated in a macromolecular carrier decorated with mannosyl residue was targeted to mannose-fucose receptors of macrophages and tested in *L. donovani*-infected macrophages. It was revealed that 50% effective dose of HPP-Rib linked to the mannosylated polylysine was less than 7.5 x 10^{-6} M, whereas it was 3 x 10' M in the case of free HPP-Rib. It was also visualized that HPP-Rib bound to the polymer was found to be 50 times more active than the free drug in killing the parasites [86].

Neo glycoproteins as a targeting tool are the cell adhesion molecules found on the cell surface and act as receptors for cell-to-cell and cell-to-extracellular matrix adhesion. These molecules are required for the efficient migration of inflammatory cells such as neutrophils and monocytes into inflamed organs and generation of the host response to infections. In this context, fucose human serum albumin (HSA) was studied to determine the characteristic recognition system involved in the receptor-mediated endocytosis. Fucose HSA illustrated strong binding affinity and uptake by the macrophages, and the binding was specific for L-fucose and D-mannose. 70% and 60% of fucose-HSA was found to be localized in liver and liver lysosomes respectively. At the same time, its uptake was 30 folds more via liver macrophages (kuffer cells) in comparison to hepatocytes. The result also suggested that fucose HSA has a greater affinity than mannose HSA for both mannose and fucose receptors and even a single type receptor [87]. In the same pathway, Chakraborty *et al.* performed binding experiments of methotrexate coupled to mannosyl-BSA. Studies indicated that conjugation did not decrease the affinity of neoglycoprotein for its cell surface receptors and was efficiently taken up by the mannosyl receptors present on the macrophages. The intracellular amastigotes of *L. donovani* in mouse peritoneal macrophages were eliminated 100 times more efficiently by the methotrexate neoglycoprotein conjugate compared to free drug. Furthermore, this inhibitory effect was found to be directly proportional to the density of sugar on the neoglycoprotein carrier. Moreover, the drug-conjugate reduced 85% of spleen parasite burden in a 30 d murine model illustrating that conjugate binds specifically to the macrophages. This may be accompanied with internalization in lysosomes and subsequent release of the active drug in the proximity of *Leishmania* parasite [88].

Promastigotes of *L enrietti* have been tested with neoglycoproteins to ascertain the existence of endogenous lectins. Agglutination test revealed that promastigotes of human *leishmania* react with neo-glycoprotein N-acetyl-D-galactosamine-para-aminophenyl-bovine serum albumin (glcNAc-BSA), since the promastigotes of *L. enrietti* were agglutinated by the D-mannose-bovine serum albumin (man-BSA) while amastigotes form failed to react. These reactions were inhibited by sugars N-acetyl-beta-D-glucosamine, N-acetyl-beta-D-galactosamine and alph-D-Mannose as was confirmed by the fluorescence tests. Results demonstrated the calcium-dependent lectins to be expressed on the surface of *leishmania* parasite and accessible to glcNAc-BSA [89]. To follow Sarkar *et al.*, (1997) exploited mannose-HSA for active targeting of muramyl dipeptide (MDP) to macrophages of VL. Mannose-HSA-MDP was found to be 50 times more effective than free MDP against *L. donovani* inside peritoneal macrophages. At the same time, a 95% reduction in spleen parasite burden in 60 d infected murine model after 4 d therapy was obtained and this was bestowed to be dependent on the physiologic generation of NO induced by IFN-gamma and TNF–alpha [90].

Scavenger receptors

Scavenger receptors are exclusively expressed on liver endothelial cells and these high binding sites are conserved on macrophages. Macrophages mediate the uptake of a variety of polyanionic proteins/ligands or macromolecular complexes. Antimony loaded liposomes (Sb-LP) made up of phosphatidylserine have been found to be preferentially taken up by macrophages scavenger receptors. Sb-LP were 16-fold more effective (IC50-514.11 mM) than the free drug (IC50-5225.9 mM) against *L. chagasi* infected macrophages. This binding and uptake of the Sb-LP were found to be energy dependent [91].

Several classes of scavenger receptors provide broad ligand specificities such as oxidized proteins and polyanions. They recognize a number of structurally diverse polyionic macromolecules such as charged modified proteins (eg. acetylated or oxidized LDL, maleylated serum albumin), polysaccharides (eg fucoidin, dextran sulphate), polynucleotide's (eg. polyguanylic and polyinosnic acids), certain acidic phospholipids (phosphatidylserine), polyvinyl sulphate and bacterial lipopolysaccharides. Methotrexate (MTX) conjugated with maleylated bovine serum albumin (MBSA) was administered to the *L. Mexicana* infected hamsters. Due to localized infection created in foot pads, 10 folds higher swelling than normal foot pads was obtained because of multiplication of protozoa. Free drug MTX exhibited no cure whereas MBSA-MTX treatment transformed the foot pads to normal size [92]. Similarly, Nieto *et al.* determined the pharmacokinetics/toxicities (in dogs) and antileishmanial efficacy (in *L. donovani* infected BALB/c mice) of plain niosomes (NIV) and dextran coated niosomes (NIV-dextran) encapsulating antimonial drug sodium stibogluconate (SSG) after administration of a single intravenous dose [51]. The NIV-dextran form significantly modified the pharmacokinetics of the drug, whereas the free drug and NIV form showed similar pharmacokinetic profile.

Monoclonal antibodies

Antibodies are highly selective for the relevant antigen, and this feature can be exploited for precise delivery of drugs to desired tissues. Moreover, it is now possible to create monoclonal antibodies, *i.e.,* antibodies designed to be specific for almost any substance, obtained from a single clone of an immune cell. They can be engineered in several ways in order to meet specific requirements from different biological environments. Antibodies are proteins composed of IgG, which contains binding fragment (Fab, responsible for specific antigen binding) and a complement-fixing fragment (Fc, responsible for fixing complement for *in vivo* biological response). Recombinant antibody technology allows for the preparation of a library of antibodies from which the ones with the required properties can be selected. Antibody fragments lacking the Fc-region, e. g., Fab′ or single-chainFv (scFv), can be used to avoid recognition by Fc receptor-bearing cells of the RES [93].

Recent developments in liposomal technology have made it feasible to investigate the therapeutic applications involving site-specific delivery mediated by antibodies. Liposomes appended with antibodies or their fragments on their surfaces are known as immuno- liposomes. Immuno liposomes have been used extensively as a drug delivery strategy towards macrophages for the treatment of VL. Anti-target antibodies having specific avidity to target has the ability to direct liposomes to the desired target. Targeting of doxorubicin to *L. donovani*-infected BALB/c mice was studied by Mukherjee *et al.* Doxorubicin was incorporated in immuno-liposomes prepared by grafting F(ab)¢2 fragment of anti-51-kDa antibody onto the liposomal surface [94]. The results showed that at a dose of 250 mg/kg/day administered for 4 consecutive days, there was a complete elimination of splenic parasite burden by doxorubicin loaded immuno- liposomes. A reduced toxicity of doxorubicin upon encapsulation in liposomal formulations was also visualized. In another approach, liposomes grafted with IgG

(immuno- liposomes) 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 FcR in macrophages. On incubation 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 [95]. Sitamaquine encapsulated PLGA-PEG nanoparticle (NP) attach with antibody to CD14 to target macrophage of infected tissues against leishmaniasis have been developed. The evaluation parameter shows significant inhibition of amastigotes in the splenic tissue with PLGA-PEG encapsulated sitamaquine as compared to the conventional (89.01±6 verse 71.39±12) [96].

Interferon-γ

Macrophage-specific targeting exploiting interferon-α may be a fascinating approach to eradicate intracellular parasites by increasing the localized manifold concentration of the drug and by reducing contraindicated manifestations resulting from systemic drug effects. In a study by Kole *et al.* (1999), the research group encapsulated doxorubicin in mannosylated liposomes and further conjugated with INF-γ. The combined chemotherapy resulted in complete elimination of splenic parasite burden. Further, mRNA levels were also analysed in infected spleen cells, and the targeted drug delivery together with IFN-a resulted in reduced levels of IL-4, increased level of IL-12 and production of inducible nitric oxide synthase. Such combination chemotherapy has proved to be a possible substitute for the cure of VL [97].

Tuftsin

Tuftsin a tetrapeptide (Thr-Lys-Pro-Arg) has been found to participate in 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 [98, 99]. The tetrapeptide enhances the phagocytic activity of monocytes and macrophages [100, 101] Specific binding sites for tuftsin have been revealed to exist on macrophages [102]. Tuftsin exclusively binds to macrophages and potentiates their natural killer activity against pathogens [103]. This makes tuftsin an attractive candidate to be used as a ligand for targeting drugs to various macrophage-related diseases. Tuftsin-based targeting of sodium stibogluconate (SSG) loaded liposomes was studied by Guru *et al.* in *L. donovani* infected hamsters for VL [104]. The findings indicated that encapsulation of SSG in tuftsin-bearing liposomes significantly enhanced the drug efficacy against L. *donovani* infection. In a similar study, the antileishmanial activity of AmB was enhanced by encapsulating the drug in liposomes. This efficacy was further increased by grafting tuftsin on the liposomal surface. The tissue distribution studies also showed higher and faster uptake of tuftsin-grafted liposomes from the circulation as compared with non-grafted liposomes since almost all the tufstin grafted liposomes were cleared within 1 hr of administration [105].

Miscellaneous factors to promote macrophage to achieve targeting

Multiple dosing

The dosing strategy is a vital parameter to achieve desired therapeutic effect. In this context multiple dose pharmacokinetics and parasite inhibition (PI) of a liposomal formulation of meglumine antimoniate in bone marrow of dogs naturally infected with the *L. chagasi* was studied. Results demonstrated an increase in antimony concentration from 0.76μg/kg to 2.07μg/kg after 4 d therapy. Though complete elimination of the parasite was not achieved yet parasite load was significantly reduced [106]. In a study by Mullen *et al.,* nonionic surfactant vesicle formulation of sodium stibogluconate (SSG-NIV) efficacy was compared with several formulations of AmB (i.e., Ambisome, Abelcet and Amphocil) in L. *donovani*-infected BALB/c mice murine model of VL. Multiple doses of AmBisome, Abelcet and Amphocil, showed different degrees of suppression in liver and spleen parasitic burden, with Abelcet having the lowest activity. AmBisome and Amphocil showed significant parasitic suppression in bone marrow. In case of acute infection model, single-dose treatments with SSG-NIV, SSG solution, or AmBisome were equally effective against liver parasites. SSG-NIV and Am-Bisome significantly suppressed parasites in bone marrow and spleen, with SSG-NIV treatment being more suppressive. On the other hand, free-SSG treatment failed to suppress spleen or bone marrow parasites. In the case of chronic infection model, the single dose AmBisome was less effective at all three sites of infection whereas single dose SSG-NIV was less effective in the spleen [50].

Polymeric conjugation

Polymer–drug conjugates have been used for passive targeting to macrophages. The strategy has exhibited potential in antileishmanial chemotherapy and modified the bio distribution of antileishmanial drugs (such as AmB) that were otherwise toxic for mammal cells when administered with water [107]. 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 [108].

Nan *et al.*, (2004) studied the bio distribution pattern and evaluated antileishmanial activity of aminoquinoline analogue, NPC1161 upon conjugation with HPMA copolymer. HPMA copolymer-NPC1161, containing N-acetylmannosamine (ManN) in the side chains, were synthesized. *In vivo* studies in *L. donovani*-infected BALB/c mice showed that HPMA-NPC1161-ManN was more effective than plain HPMA-NPC1161 conjugates [70]. HPMA copolymers containing ManN in the side chains could potentially reduce the toxicity and increased the efficacy of antileishmanial drugs for the treatment of VL. Similarly, Kozan and co-workers (2002) coupled Methotrexate (MTX) with different synthetically branched polypeptides such as poly [Lys (DL-Alam)](AK), poly[Lys(Seri-DL-Alam)](SAK), poly[Lys (DL-Alam-Leui)](ALK) and poly[Lys(Glui-DL-Alam)] (EAK). Midest of these conjugates, MTX-ALK produced the most encouraging data, with 95% parasitic inhibition in the liver as compared with free MTX (42% PI) when 5 injections (100 μg of MTX/injection) were administered intraperitoneally. Furthermore, the covalent bond between the carrier and the drug was observed to be crucial for its activity [109].

CONCLUSION

The drug discovery pipeline for the treatment of leishmaniasis is imbalanced and still requires improved control tools. Along with efforts to find new compounds, resources availability is essential at this crucial stage of drug development. Novel therapies are the current hopes and have been promising with the pioneering of AmBisomes, for the treatment of VL. The formulation exhibits reduced toxicity, shorter treatment period and effective response to a single dose; but limits itself to a fraction of the population owing to its unaffordable cost. With advances in the development of drug delivery systems, a new era in the treatment of leishmaniasis has begun. The use, of drug carriers (i.e., liposomes, niosomes, emulsions, micro/nano particles) to efficiently deliver the antileishmanial agents inside the cells infected with *leishmania* parasite has been supported by numerous studies and are able to modify the distribution of an associated drug substance. 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. However, owing to their complexities, site directed drug delivery systems such as ligand directed carriers undoubtedly cost more in development and manufacturing than conventional therapeutic agents. The resulting higher prices may be acceptable if the increase in cost can be minimized and if the performance of these drug delivery systems is increased sufficiently thereby reducing the treatment period and cost to produce overall savings to the healthcare system.

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CONFLICT OF INTERESTS

The authors have no conflict of interest to declare.

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