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Research Article

Development and Evaluation of P-Aminophenylmannopyranoside Anchored Emulsomes for Treatment of Experimental Visceral Leishmaniasis

Navneet Kumar[1], Prachi Sharma[2], Anil Jaiswal[3], Anuradha Dube[3], and Swati Gupta[1,2]*

[1]Department of Pharmaceutics, Nanomedicine Research Center, India
[2]School of Pharmaceutical Sciences, Apeejay Stya University, India
[3]Division of Parasitology, CSIR-Central Drug Research Institute Lucknow, India

*Corresponding author
Swati Gupta, School of Pharmaceutical Sciences, Apeejay Stya University, Sohna-Palwal Road, Sohna-122103, Gurgaon, Haryana, India, Tel: 9582699771; Email: swatig25@gmail.com

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Abstract

Amphotericin B (AmB) was formulated in tripalmitin based nanosize lipid particles (emulsomes) stabilized by egg phosphatidylcholine (PC) for macrophage targeting for the treatment of visceral leishmaniasis (VL). Emulsomes were prepared by cast film technique followed by sonication to obtain particles of nanometric size range and further modified by anchoring them with macrophage-specific ligand (p-aminophenyl-α-D-mannoside, PAM). The surface modified emulsomes and their plain counterparts were characterized for size, shape, entrapment efficiency, zeta-potential, in-vitro drug release and ligand binding specificity. The antileishmanial activity of AmB-deoxycholate (AmB-Doc) and emulsome entrapped AmB was tested *in vitro* in *Leishmania donovani* infected macrophage-amastigote system (J774A.1 cells), which showed higher efficacy of PAM-anchored AmB emulsomes (TPEs-PAM) over plain AmB emulsomes (TPEs) and AmB-Doc. The *in vivo* antileishmanial activity of the AmB (0.5 and 1mg/kg respectively) was tested against VL in *L. donovani* infected hamsters in which formulation TPEs-PAM eliminated intracellular amastigotes of *L. donovani* within splenic macrophages more efficiently (52.44 ± 3.9 % and 68.03 ± 4.2 % parasite inhibition, PI) than the formulation TPEs (32.86 ± 2.4 % and 39.66 ± 3.8 % PI) and AmB-Doc (20.98 ± 2.10 % and 24.43 ± 3.55 % PI). We concluded that PAM-anchored emulsomes could fuse with the macrophages of liver and spleen due to ligand-receptor interaction and could target the bioactives inside them. Our results suggest that these newer formulations (plain and ligand-anchored emulsomes) are a promising alternative to the conventional AmB-Doc formulation for the treatment of VL.

ABBREVIATIONS

VL: Visceral leishmaniasis; AmB: Amphotericin B; PAM: p-aminophenylmannopyranoside; PE: Phosphatidylethanolamine; Con-A: Concanavalin-A

INTRODUCTION

Visceral leishmaniasis (VL) results from infection of the macrophages of the liver, spleen and bone marrow with protozoal parasite *Leishmania donovani*. India alone may contribute as much as 40–50% of these with 90% occurrence in the state of Bihar [1,2]. WHO model list of essential medicines 14th edition (March 2005) enlists only antimonials for the treatment for VL, but there is emerging evidence that the rates of response to the antimonials are declining due to the appearance of resistance, and relapses are common [3]. Amphotericin B (AmB) provides substantial leishmanicidal activity as well, and its use results in fewer treatment failures and relapses. However, the important side effects, mainly nephrotoxicity, produced by this drug at therapeutic doses have often led to its refusal as a first-choice treatment [4].

For diseases of microbial etiology, 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 [5]. One such approach to increase the efficacy and to reduce the dose related toxicity of these agents is to target the drug molecule to the phagolysosomes of the MPS where the leishmania parasites reside [4,6]. Thus, there is an urgent need to develop safe drug-delivery strategies for existing molecules. Novel drug delivery systems, such as liposomes, microspheres and nanospheres can result in higher concentrations of AmB in the liver and spleen but lower concentrations in the kidney and lungs, thus decreasing the toxicity of AmB. The ability of nanocarriers to be taken up by MPS makes them ideal vehicle for selective transport of drug to target tissues in disease where phagocytic cells are involved.

However, most of these colloidal carriers fail to deliver the drug to diseased area efficiently due to nonspecific MPS uptake and poor target specificity. The natural passive uptake suffers from many inherent disadvantages like poor drainage at the site of injection and nonhomogeneous distribution to various

macrophages-specific tissues.

Surface modification of these carriers with site-specific ligands further facilitates their rate and extent of macrophage accumulation of drug which may further reduce the dose size and dose frequency of already licensed lipid AmB formulations for the treatment of VL [7]. The exclusive presence of mannose/fucose receptors on macrophages has been exploited for developing an efficient macrophages-directed drug carrier [8,9].

AmB is a potent polyene antibiotic, available as a micellar solution (Fungizone®), liposome (AmBisome®), AmB lipid complex (Abelcet®), and AmB colloidal Dispersion (Amphocil®) for effective treatment of VL and some other systemic fungal infections like candidiasis. However, the utility of these new products is greatly limited due to their high costs. So there is a need for the development of low cost formulations [10].

The objective of the proposed work is the effective and site-specific localization of AmB inside the macrophages to treat the VL using emulsomes. Emulsomes are a new generation colloidal carrier system in which internal core is made of fats and triglycerides which is stabilized by high concentration of lecithin in the form of o/w emulsion. By virtue of solidified or semisolidified internal oily core it provides a better opportunity to load lipophilic drugs like AmB in high concentration, simultaneously a protracted controlled release can also be expected. Also, due to their colloidal nature, they can be passively taken up from the blood stream by the macrophages of the liver and spleen (host for the leishmania parasite) after intravenous or intracardiac administration [11] and hence can be very useful for the treatment of VL. 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 current commercial lipid AmB formulations.

In our previous work, we have developed mannosylated emulsomes and solid lipid nanoparticles for macrophage targeting using AmB against VL [10,11]. In the present study, we have developed emulsomes using different lipid and coated them with another macrophage specific ligand, p-aminophenylmannopyranoside (PAM) to check its antileishmanial efficacy and the results of free AmB, plain and ligand-anchored emulsomes entrapped AmB are compared.

MATERIALS AND METHODS

Drugs and chemicals

Amphotericin B was a kind gift from Life care Innovations Pvt. Ltd., Gurgaon, India. AmB-deoxycholate (AmB-Doc, Amphotret™) was purchased from Bharat Serums and Vaccines Limited., Ambernath, India. Egg phosphatidylcholine (PC), phosphatidylethanolamine (PE), cholesterol, p-aminophenylmannopyranoside (PAM), concanavalin A (Con-A) and Sephadex G-50, Dialysis tubing (Mw 12 kDa) were purchased from Sigma Chemicals Company (St Louis, MO, USA). Tripalmitin, glutaraldehyde and trichloroacetic acid were purchased from Himedia Laboratories Pvt Ltd., Mumbai. Potassium dihydrogen phosphate, disodium hydrogen phosphate, and mannitol were purchased from Rankem Laboratory Reagents, New Delhi. Chloroform and all other chemicals were of pure analytical grade and used as procured.

Parasite

The WHO reference strain, *L. donovani* (MHOM/IN/80/Dd8) promastigotes were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), penicillin (100 U/ml) and streptomycin (100µg/ml) at 268C. Parasites were also maintained through *in vivo* serial passage (amastigote to amastigote) in hamsters [12].

Animal host

Laboratory bred male Syrian golden hamsters (*Mesocricetus auratus*, 45–50 g) from animal house facility of Central Drug Research Institute (CDRI) were used as the experimental host. They were housed in plastic cages in climatically controlled rooms and fed with standard rodent food pellet (Lipton India Ltd, Bombay) and water *ab libitum*.

Preparation of emulsomes

Emulsomes containing AmB were prepared following the already established and reported method of our laboratory [11]. To a 250 ml round-bottomed flask, AmB was dissolved in methanol by sonication. In a separate beaker PC, tripalmitin and cholesterol were codissolved in chloroform. Both organic solutions were mixed, and the organic solvent was evaporated until complete dryness under reduced pressure using a rotary flash evaporator in to form a thin lipid film on the walls of the round-bottom flask. The dried film was hydrated with phosphate-buffered saline (PBS) pH 7.4 (4 ml) and homogenized by ultrasonication at 50% amplitude to obtain emulsomes of nanometric size range. The free un-entrapped drug was removed by passing the dispersion through a sephadex G-50 column [13].

Optimization of emulsomes

Emulsomes were optimized for AmB to lipid ratio, PC to tripalmitin ratio and sonication time. For optimization of AmB to lipid ratio, PC to tripalmitin ratio (1:1 w/w), cholesterol (4% w/w of PC) and sonication time (4 min) were kept constant while AmB content was varied at different weight percent ratio levels, i.e. (1, 2, 4, 6, 8% weight of the lipid) in different formulations (Table 1) for determining optimum AmB content. Average particle size of different formulations was determined by particle size analyzer (Delsa NanoC, Beckman Coulter Pvt. Ltd., USA) and percent drug entrapment in different formulations was also determined as described previously [16]. Emulsomes with optimum AmB to lipid ratio were optimized for optimum sonication time in terms of average particle size. PC to tripalmitin ratio (1:1w/w), cholesterol (4% w/w of PC) and AmB to lipid ratio (optimized) were kept constant while sonication time was varied (i.e. 0, 2, 4, 6, 8 min) for different formulations. Emulsomes with optimum AmB to lipid ratio and sonication time were optimized for optimum PC to trilaurin ratio in terms of percent drug entrapment and toxicity towards erythrocytes. The emulsomes with fixed cholesterol (4% w/w of PC) and different PC to tripalmitin ratios (0.6:1, 0.8:1, 1:1, 1.2:1, 1.4:1 w/ ratios) were prepared. AmB content and sonication time however were kept constant at its optimum level. Emulsomes were evaluated for percent drug entrapment and toxicity to mammalian cells in terms of percent haemolysis.

Table 1: Optimization of AmB to lipid ratio.

S.No	Formulation code	AmB content [a]	Average particle size (nm)	Polydispersity index	% Entrapment Efficiency
1	TPE1	1	1289.7 ± 1.9	0.417 ± 0.02	89.87 ± 2.01
2	TPE2	2	1082.4 ± 2.1	0.344 ± 0.03	87.17 ± 1.85
3	TPE3	4	489.0 ± 1.0	0.176 ± 0.01	82.37 ± 0.96
4	TPE4	6	286.2 ± 1.5	0.347 ± 0.02	70.14 ± 1.81
5	TPE5	8	153.0 ± 2.0	0.225 ± 0.03	63.80 ± 2.86

Total lipid used 40 mg; PC: tripalmitin = 1:1 w/w ratio; Cholesterol = 4% w/w of PC; Sonication time = 4 min at amplitude 50; pulse on time: 8 sec, pulse off time: 5 sec for each formulation (mean ± S.D.) (n=3), [a]Percent w/w ratio of AmB to lipid.
Abbreviations: AmB: Amphotericin B; PC: Phosphatidylcholine.

Percent haemolysis was determined by the method reported in literature [7]. Percent haemolysis and percent drug entrapment were plotted against PC to tripalmitin ratio, from which optimum PC to tripalmitin ratio was determined.

Preparation of p-aminophenyl-mannopyranoside (PAM)-anchored emulsomes

For the preparation of PAM- anchored emulsomes, firstly phosphatidylethanolamine (PE)-containing emulsomes were prepared by incorporation of PE as one of the phospholipids at 10% w/w of PC using optimized formulation of emulsomes [11]. Now, mannosylated ligand (PAM) was inserted into the lipid bilayer using the PE end groups (Figure 1). PAM was linked to PE -containing emulsomes by the method described earlier [14,15]. A 2.5 ml of PE-emulsome dispersion (~30 mg lipids) in PBS (7.4) was mixed with 20.0 mg PAM contained in 2.0 ml of 0.9% w/v aqueous NaCl solution. Glutaraldehyde (0.5 ml of 25% v/v aqueous solution) was then slowly added to the dispersion to a concentration level 3 mM and the mixture was incubated for 5 min at 20°C. The -NH_2 group of PE-emulsomes was coupled with p-aminophenyl-mannopyranoside using glutaraldehyde as coupling agent [15]. Uncoupled glycosides (sugar derivatives) and glutaraldehyde were removed through dialysis using dialysis membrane (12 kDa) technique against the same buffer (Figure 1).

In-vitro characterization

Developed formulations were characterized before and after surface ligand anchoring. Prepared emulsomes were evaluated for their shape by both transmission electron microscopy (TEM) (Hitachi, Japan) and scanning electron microscopy (SEM) (JEOL, EVO-50 Japan). A drop of the sample was placed on to a carbon coated copper grid to leave a thin film on the grid. Phosphotungstic acid (1%) was used as a negative stain. A drop of the staining solution was added on to the film and the excess of the solution was drained off. The grid was allowed to dry at ambient temperature and subjected to TEM analysis. Size, polydispersity index (PDI) and zeta-potential was determined by photon correlation spectroscopy method using Bechman Coulter Zetasizer (NanoDelsaC; Bechman Coulter Pvt. Ltd., UK).

Percent drug entrapment was determined and expressed as the ratio of experimentally measured amount of drug in dispersion and initial amount used for entrapment. Centrifugation was done at 3000 rpm for 3 min to remove unentrapped drug. Vesicles (free of unentrapped drug) were lysed by adding 1.0 ml of 0.1% v/v triton X-100 and liberated contents were analyzed for AmB content spectrophotometrically at 405 nm [5].

In-vitro drug release

The *in-vitro* drug release profiles of AmB from different emulsomal formulations were determined using a dialysis tube (MWCO 12 kDa; Sigma Chemical Co., USA) method. A 2 mL volume of the formulation was taken in the dialysis tube (donor compartment) and placed in a beaker containing 100 ml of PBS/DMSO (95%:5% v/v) (receptor compartment). The assembly was placed over a magnetic stirrer at 100 rpm and the temperature was maintained at 37 ± 1°C throughout the study. Samples (5 ml) were withdrawn periodically and after each sample withdrawal the medium was compensated immediately with fresh dialyzing medium PBS/DMSO (95%:5% v/v) while maintaining strict sink conditions. The samples were analyzed for AmB content spectrophotometrically at 405 nm [4].

In vitro ligand binding specificity

In vitro ligand-specific activity was performed to assess the surface orientation and availability of accessible mannose residues of PAM after the formation of PAM-anchored emulsomes using concanavalin-A (Con-A) lectin as reported with slight

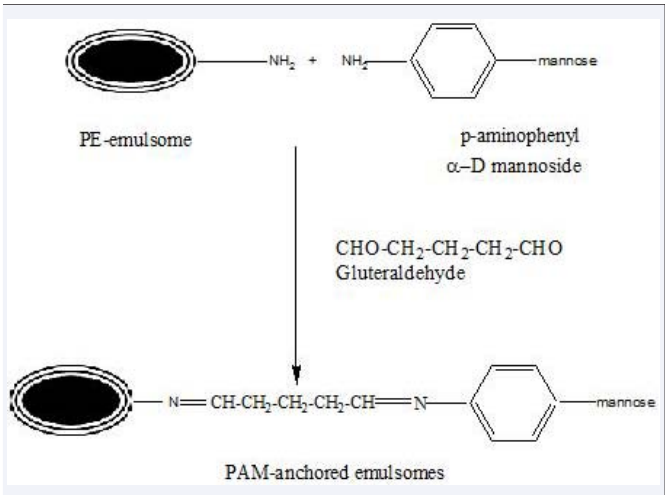

Figure 1 Coupling of p-aminophenyl-α-D-mannoside with emulsome.

modification [16]. The affinity towards exogenously provided lectin Con-A was used as a measure of activity of PAM-anchored emulsomes. A 200µL sample of the original emulsomal dispersion (both plain and PAM-anchored) was diluted 10-times with PBS (pH 7.4) and 1mL Con-A (1mg/ml) in PBS (pH 7.4) with 5mM $CaCl_2$, and 5mM $MgCl_2$ was added to it. The increase in turbidity at 550 nm was monitored spectrophotometrically for 2 h.

In-vitro activity against intracellular amastigotes in macrophages

Mouse macrophage adherent cell line J774A.1 was maintained in RPMI-1640 medium (Sigma, USA) supplemented with 10% heat inactivated fetal bovine serum (HIFBS) at 37˚C in humidified atmosphere of 5% CO_2. Macrophages (10^5 cells/well) in 16-well chamber slides (Nunc, IL, USA) were infected with promastigotes (*L. donovani, Dd8*) at multiplicity of infection of 10:1 (parasites/macrophage) and incubated at 37˚C in 5%

CO_2 for 12 h after which the chamber slides were washed thrice with PBS (pH 7.2) to remove non-phagocytosed promastigotes and finally supplemented with complete medium [17]. Different concentrations (0.08 and 0.16µg/ml) of 100 µl of free AmB (AmB-Doc, Mycol®), optimized tripalmitin emulsome formulations (TPES4P4), and PAM-anchored emulsome formulations (PAM-TPEs) or (TPES4P4-PAM) in RPMI-1640 medium were added to wells in triplicate. Similarly empty TPES4P4 and empty TPES4P4-PAM without AmB but having same composition in RPMI-1640 medium were added to wells in triplicate. The untreated infected macrophages were used as control. Formulations were then removed by washing after 3 h and macrophages were placed in medium for an additional 20 h, after that they were examined for intracellular amastigotes under oil immersion objective of light microscope after methanol fixing and Giemsa staining of the slides. At least 100 macrophage nuclei were counted per well for calculating percentage infected macrophages and number of amastigotes per 100 macrophages. Percent parasite inhibition in treated wells was calculated using the following formula [10,17].

$$\mathbf{PI} = \frac{\mathbf{100} - T \mathbf{\times 100}}{C}$$

Where PI is the percentage inhibition, T is the number of parasites in treated samples/100 macrophage nuclei, and C is the number of parasites in control samples/100 macrophage nuclei.

Stability studies

Stability in serum: The stability of emulsomes in the serum was determined by observing drug leaching following incubation of emulsomes with freshly pooled rat serum at 37 ± 1 ºC. The drug content of the emulsomes was determined by the method described [11]. Emulsomes formulations (1.0 ml) (both plain and PAM-anchored) were incubated with 2 ml serum at 37 ± 1ºC for 1, 2, 4, 6 and 24 h. After specified time intervals, suspensions were centrifuged at 18,000 rpm for 20 min and supernatant was filtered through 0.45 µm membrane filter. The filtrate was analyzed for drug content by measuring absorbance at 405 nm against the blank using UV-1700 spectrophotometer.

Effect of storage on size and percent residual drug content: Storage stability of optimized formulations (both plain and PAM-anchored) was carried out after storing them in tightly closed container at 37 ± 1ºC, 28 ± 1ºC and 4 ± 1^0C for a period of 30 days. The samples were analyzed for change in average particle size by using zeta sizer. The optimized emulsomes formulations were stored at 37 ± 1ºC, 28 ± 1 ºC and 4 ± 1^0C and percent residual drug content was evaluated after different time intervals i.e. 10, 20 and 30 days. Samples were taken periodically and estimated for drug content as described earlier [11].

In-vivo studies

Developed formulations were studied for antileishmanial activity in Syrian golden hamsters at Divison of Parasitology (CDRI, Lucknow). The study protocol for *in vivo* organ distribution studies was approved by Institutional Animals Ethical Committee (Ref: ISF/IAEC/35/10). The study was carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

***In vivo* serum and organ distribution studies:** Male albino rats (water and food ab libitum) weighing about 150-200 g were divided in three groups of 12 rats each. Free AmB or AmB-Doc (Mycol®, 1 mg/kg body weight) and emulsome (both plain and PAM -anchored) containing equivalent doses of AmB (1mg/kg body weight) were administered intraperitonealy to different groups. Animals from each group were sacrificed at 1, 4, 10 and 24 h after administration of the formulations. Blood samples were collected by cardiac puncture. Visceral organs (liver, spleen, lung and kidney) of the dissected rats were excised, isolated, washed with distilled water and were blot dried using tissue papers [5]. After drying these were weighed separately and were homogenized with 2 ml of PBS (7.4) using tissue homogenizer. The tissue homogenates were deproteinized with an equal volume of 10% v/v trichloroacetic acid, kept in dark for 30 min, centrifuged at 5000 rpm for 5 min to precipitate tissue proteins. After precipitation of proteins, supernatant was collected. One mL of collected supernatant was filtered through a 0.45-µm filter into a 10-mL volumetric flask and volume was made up with the methanol. The serum was harvested from collected blood samples, deproteinized and processed in a similar way. The filtrates were analyzed for AmB content using UV-spectrophotometer at 405 nm. The amount of drug in each organ was calculated as percent drug recovered from the respective organ at different time intervals [5].

***In vivo* antileishmanial activity testing:** The isolation of parasites (*L. donovani, Dd8*) and infection to naive hamsters (45-50g) were carried out as described earlier [10]. Briefly, the infected animals (*L. donovani, Dd8*) carrying 45-60 day old infection were autopsied, their spleen removed aseptically, homogenized in Locke's solution (8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.3 g KH_2PO_4 and 2.5 g glucose in 1L , pH 7.2) and centrifuged at 900 rpm for 5 min at 4˚C to sieve out tissue debris. Supernatant was centrifuged at 2,500 rpm for 10 min. The pellet was washed twice with PBS and resuspended to obtain a concentration of $1x10^7$ amastigotes per 0.1 ml of PBS and these inoculums was injected to each hamster intracardially (i.c.). After 25 days, infection was confirmed by Giemsa staining of tissues smears of spleen after the biopsy of two animals. Infected animals harboring 38-40 amastigotes/100 macrophage nuclei were then distributed (six in each group) for drug treatment in the following manner: (i) Infected controls (no

therapy was given), (ii) Free AmB (AmB-Doc, Mycol®) treated, (iii) TPEs (TPES4P4) treated, (iv) PAM –TPEs (TPES4P4-PAM) treated, (v) Empty TPEs (TPES4P4) (without AmB) treated, (vi) Empty PAM –TPEs (TPES4P4-PAM) (without AmB) treated.

Chemotherapy was performed following the method as described [17] with slight modifications. For the purpose of chemotherapy 0.05 mg of the drug (AmB) intercalated in 0.1 ml of emulsome suspension (equivalent to 0.5 mg/kg) and 0.1 mg of the drug (AmB) intercalated in 0.2 ml of emulsomes suspension (equivalent to 1 mg/kg) was injected intraperitoneally (i.p.) into each hamster on day 31st and 33rd post infection. For free drug treatment the AmB-Doc in PBS was injected each time at the same dose. Splenic biopsies were again performed on day 7-post treatment (p.t.) after administration of the last dose of each formulaion. Parasite burden in both treated and untreated infected animals was assessed by counting number of amastigotes in Giemsa stained splenic smears and percent parasite inhibition in treated animals was calculated using the following formula:

$$PI = \frac{100 - AN \times 100}{INA \times TI}$$

where PI, percent inhibition; AN, the actual number of amastigotes/100 spleen cell nuclei in treated animals; INA, the initial number of amastigotes/100 spleen cell nuclei in treated animals; TI, fold increase in the number of amastigotes in untreated control animals on the corresponding day of the biopsy in treated animals.

Statistical analysis

Results were expressed as mean ± SD. Three sets of experiments were performed. The data were statistically processed by one-way analysis of variance (Graph Pad; Prism software program) followed by post-hoc Turkey's multiple comparison test (n=3) to determine the level of significance. Differences were considered statistically significant at $P < 0.05$.]

RESULTS AND DISCUSSION

Preparation and *in vitro* characterization

The study opted here was designed for the development and characterization of model antifungal and antileishmanial drug, AmB bearing emulsomes, which are lipid particles with an internal solid fat core composed of solid lipid surrounded by phospholipid bilayers in the form of intermediate stage between liposomes and o/w emulsion [17] in an attempt to overcome the nephrotoxicity and other side effects of AmB for the treatment of VL.

Emulsomes formulations, based on PC and tripalmitin as the constitutive lipids, were prepared. The drug AmB was incorporated into the emulsomes using cast film method. Emulsomes were optimized for various parameters. These include the weight ratio of AmB to total lipid, sonication time and ratio of PC to tripalmitin. At higher concentration, (6 and 8 weight % of the total lipid) of AmB, entrapment efficiency and particles size were found to be lower (70.14 ± 1.81 and 63.80 ± 2.86 % and 286.2 ± 1.5 and 153.0 ± 2.0 nm respectively). It may be attributed to the leaky membrane formations due to associations of AmB molecule with cholesterol, which may result in lower entrapped volume and hence lower size of the particles. This hypothesis however, needs confirmation from experimental studies [5]. When concentration of AmB was used at 4% weight of total lipid, average particle size measured was 489.0 ± 1.0 nm and entrapment efficiency was recorded to be 82.37 ± 0.96%. Below this concentration, (i.e. 2 and 1% weight of AmB to total lipid) percent drug entrapment as well as particle size were recorded to be increased (87.17 ± 1.85 and 89.87 ± 2.01% and 1082.4 ± 2.1and 1289.7 ± 1.9 nm respectively) (Table 1, Figure 2). But formulation having AmB concentration 4% weight of total lipid (formulation TPE3) was considered to be optimum because for intravenous or intracardiac administration purpose, particle size should be below 1 µm to prevent formation of emboli in the blood vessels [17] and formulation TPE3 is having particle size in this range. Formulation with optimum AmB to lipid ratio was subjected to sonication for different time periods to optimize the sonication time. Figure 3 shows that as the sonication time was increased from 0 to 8 min, average particle size was recorded to be decreased. It is already reported that particulates having size of 0.2–7 µm clear rapidly from circulation via macrophages however smaller particles (100–200 nm) escape through fenestrae and localize in hepatocytes [4]. From this hypothesis,

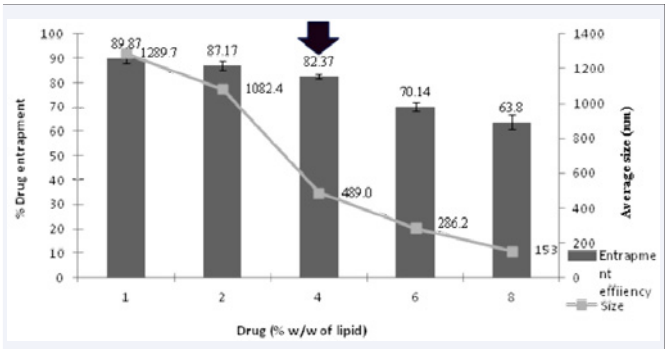

Figure 2 Optimization of AmB to lipid ratio in terms of average particle size and % entrapment efficiency.

Total lipid used 40 mg; PC: tripalmitin - 1:1 w/w ratio; Chol - 4% w/w of PC; Sonication time - 4 min at amplitude 50; pulse on time: 8 sec, pulse off time: 5 sec for each formulation (mean ± S.D) (n=3); a Percent w/w ratio of AmB to lipid.

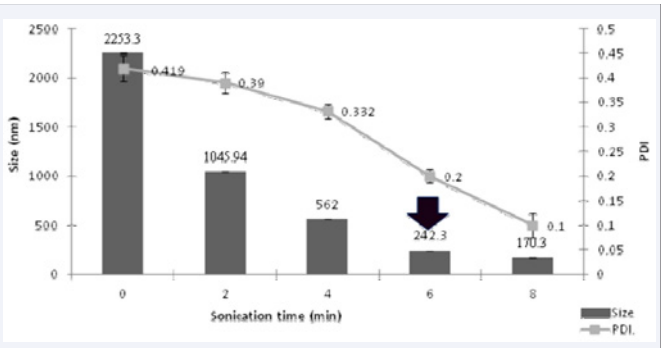

Figure 3 Optimization of sonication time in terms of average particle size and polydispersity index.

Total lipid used 40 mg; Amp-B- 4% w/w of lipid, PC: tripalmitin - 1:1 w/w ratio; Chol - 4% w/w of PC; Amplitude 50; pulse on time: 8 sec, pulse off time: 5 sec for each formulation (mean ± S.D) (n=3).

optimum sonication time was recorded to be 6 min, which gave particle size of 242.3 ± 0.75 nm. On further increasing the sonication time (i.e. at 8 min) beyond the optimum limit, the particle size was recorded to be 170.3 ± 1.52 nm and emulsomes might have localized in hepatocytes apart of macrophages, the target site (Table 1, Figure 2,3).

Formulation having optimized AmB to lipid ratio and sonication time was subjected to optimization of PC to tripalmitin ratio. Figure (4) shows that with an increase in PC concentration in emulsomes, distinctive changes in toxicity to the erythrocytes and percent entrapment of AmB were recorded. As the PC: tripalmitin w/w ratio was increased from 0.6:1 to 1.4:1, percent drug entrapment was also recorded to be increased (Figure 4). This may be attributed to the formation of more bilayers upon increasing the concentration of PC and intercalation of AmB in the bilayers also apart of internal solid lipid core. Similarly as the ratio of PC: tripalmitin was increased, percent haemolysis was also recorded to be increased (Figure 4).

After optimizing the process parameters, PE-emulsomes were anchored with macrophage specific ligand PAM. Since PAM is having free $-NH_2$ group on its para- position, it can be simply anchored to the lipid bilayer using the PE end groups which employs the use of glutraldehyde which acts as a coupling agent between these two, with simple incubation. It was necessary to use glutaraldehyde, which allowed the surface anchoring and inter-digitization of PAM to emulsomes membrane. During the anchoring process, the $-NH_2$ group of PAM integrated with the lipoidal emulsomes membrane projecting from the hydrophilic mannose residues towards the bulk aqueous phase. Structure of PAM– anchored emulsomes was confirmed by FT-IR, spectroscopy (Results not shown). IR spectrum shows peak in the region of 3457.31 cm^{-1}, which is characteristic O-H stretching peak providing evidence for polyhydroxy groups of mannose; C=N stretching peak is observed at 1635.72 cm^{-1} providing evidence for anchoring of glutraldehyde and NH_2; C-O stretching shows peak at 1106.9 cm^{-1} which confirms the alcoholic group of mannose. All the peaks in the IR spectra provided convincing evidences for the existence of PAM anchoring on emulsome surface.

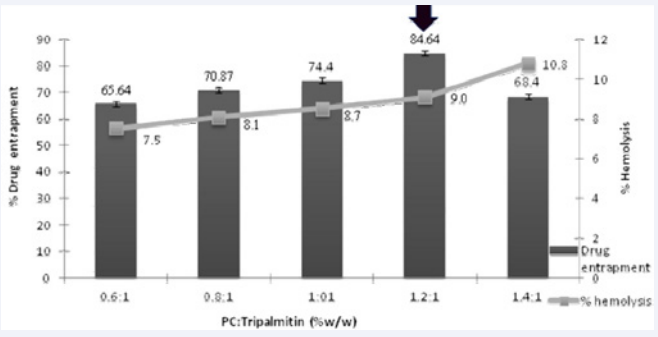

Figure 4 Optimization of PC content in terms of percent drug entrapment and percent haemolysis.

Total lipid used 40 mg; Amp-B- 4% w/w of lipid, Cholesterol - 4% w/w of PC; Sonication time - 6 min at amplitude 50; pulse on time: 8 sec, pulse off time: 5 sec for each formulation (mean ± S.D) (n=3).

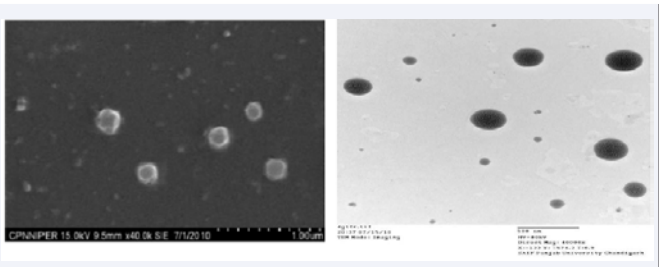

Figure 5 (A) SEM photomicrograph of plain emulsomes. (B) TEM photomicrograph of plain emulsomes.

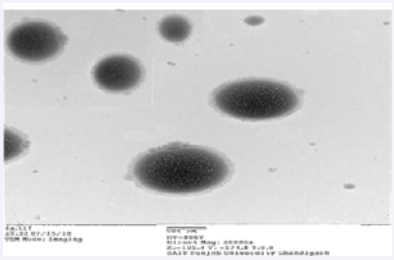

Figure 6 TEM photomicrograph of PAM –anchored emulsomes.

The shape and surface morphology of the emulsomes were determined by TEM and SEM. The SEM and TEM photographs suggested that the emulsomes are smooth, spherical, uniform and multilamellar in nature (Figure 5A, 5B). Anchoring of PAM on the emulsomes surface could also be appreciated from the TEM, which indicates surface anchoring and interdigitization of mannose terminating ligand (Figure 6, 4, 5A, 5B, 6).

Table (2) shows the average particle size, polydispersity index, zeta-potential and percent drug entrapment of optimized plain and PAM -anchored emulsomes. The increase in average particle size in case of PAM –anchored emulsomes (380.0 ± 1.09 nm) as compared to plain emulsomes (218.7 ± 1.03 nm) is an indication of anchoring, which can be distinguished by thick boundary of the emulsomes (Figure 6). Percent drug entrapment of optimized plain and PAM –anchored emulsomes was found to be 84.40 ± 1.05 and 82.20 ± 1.88 %, respectively, which revealed that PAM anchoring did not result in significant lowering of the percent drug entrapment (Table 2). Preformed emulsomes were used for anchoring of ligand and this may presumably be the reason for the insignificant change recorded in the percent drug entrapment value. Relatively high entrapment of AmB in the emulsomes could be attributed to the lipophilic nature of the drug, since the entrapment was dependent upon lipid: aqueous phase ratio. Due to the lipophilic nature of the AmB, it probably gets intercalated preferentially into the solid lipid core as well as into the multilamellar vesicle lipid domains. In addition, PAM – anchored emulsomes were characterized by zeta potential, which further confirmed the surface availability of PAM as significant reduction in zeta potential was observed (Table 2). Also emulsomes were positively charged due to the phosphatidylethanolamine, which might facilitated the adsorption process of PAM and resulted in a reduction of the zeta potential of the dispersion. The zeta potential of AmB-loaded emulsomes, that is, plain and PAM –

Table 2: FT-IR spectral illustrations of PAM –anchored emulsomes.

Wave number (cm^{-1})	Functional group	Interpretation
1106.9	C – O stretch	Alcoholic group of mannose
1635.72	C = N stretch	Anchoring of glutraldehyde and NH_2
3457.31	O – H	Polyhydroxy group of mannose

anchored emulsomes, was found to be -15.70 ± 1.05 and -28.13 ± 1.02, respectively (Table 2).

In-vitro release profiles of the drug from plain and PAM-anchored emulsomes was determined in PBS/DMSO (95%:5% v/v; pH 7.4) for a period of 60 h. The cumulative percent drug released from the plain and PAM-anchored emulsomal formulations was characterized by an initial burst release of approx 8 ± 1.7 and 7.0 ± 1.8% in the first hour, followed by controlled release. The percentage of drug released after 60 hrs was found to be 58.5 ± 1.4 and 52.0 ± 1.8% respectively (Figure 7A,7B). In prepared emulsomes initial fast release in 1st hr might be due to the release of drug from PC bilayers surrounding the lipid core and later on prolonged release could be attributed to the release of drug from the solid lipid core. The drug released after 60 hrs in PAM anchored emulsomes was slightly less as compared to plain emulsomes. This might be attributed to the hindrance of drug release offered by the PAM anchoring over the emulsomes [10] (Figure 7A,7B).

Con-A, a lectin from Jack Bean (*Canavalia ensiformis*), is a well-investigated lectin having a tetrameric protein with four binding sites. It binds specifically with saccharides, such as mannose, fructose, and glucose residues. The presence of accessible mannose residues on the vesicle surface was confirmed by *in-vitro* Con-A. Con-A is one of the well-investigated lectin and is known to specifically bind mannose residues [18]. A dramatic increase in turbidity as monitored by taking absorbance at 550 nm in the case of dispersion containing PAM-anchored emulsomes following exposure to Con A. In contrast, plain emulsomes did not show any significant change ($P < 0.05$) in turbidity following exposure to lectin (Figure 8). The results suggested that PAM retained the binding specificity towards the lectins even after chemical modification and anchoring onto the emulsome's surface, and they were oriented towards the aqueous bulk and were available for such interaction (Figure 8).

In vitro activity against intracellular amastigotes in macrophages

The activity of different concentrations of AmB in various formulations against *L. donovani* (*Dd8* strain) was assessed in macrophages (J774A.1). During the assessment of tripalmitin emulsomes, AmB-Doc showed 10 ± 4.5 and 17.6 ± 5.3 % parasite inhibitions at the doses of 0.08 and 0.16 µg/ml respectively. Formulation AmB-TPEs showed better efficacy as compared to AmB-Doc with 39.2 ± 4.9 and 45.4 ± 5.5 % parasite inhibitions at the doses of 0.08 and 0.16µg/ml respectively (Figure 9). Formulation AmB-TPEs-PAM was found to be most effective of all drug forms and showed 51.57 ± 4.8 and 67.0 ± 5.9% parasite inhibitions at the doses of 0.08 and 0.16µg/ml respectively. The significantly higher ($P < 0.01$) efficacy of AmB-TPEs-PAM as compared to AmB-Doc may be attributed to the facilitated delivery of the drug (in the form of ligand anchored emulsomes) to macrophages through mannose receptors as well as prolonged presence of drug inside the macrophages in the form of carriers [17]. The empty TPEs and TPEs-PAM formulation also showed 7.84 ± 3.2 and 9.04 ± 3.9% parasite inhibition respectively (data not shown in figure) which was significantly lower ($P < 0.001$) as compared to AmB-Doc and drug loaded similar formulations (i.e. TPEs and TPEs-PAM). This low level of inhibition may arise through the release of hydrogen peroxide following the activation of macrophages [10] (Figure 9).

Stability in serum

Stability of developed emulsomes in serum was measured as % drug leaching from emulsomes after incubation with serum at 37 ± 1ºC for different time periods. The emulsomes were found to be almost stable upon incubation with freshly pooled rat serum. Only 6.9 ± 0.38 and 4.7 ± 0.35 % drug was leached into serum after

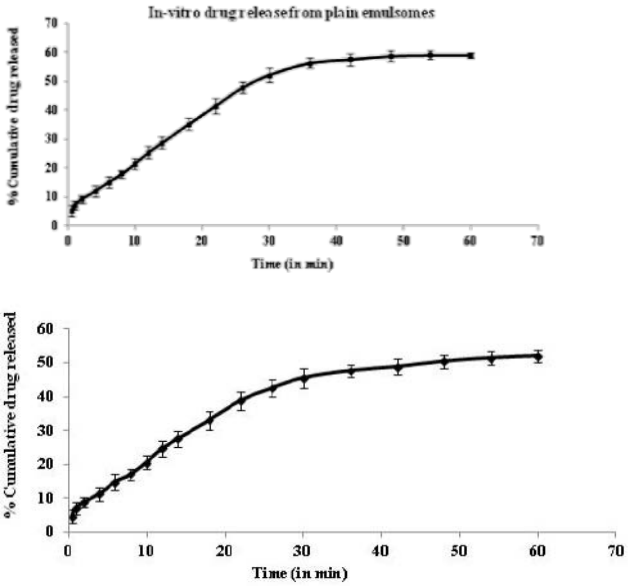

Figure 7 (A) In-vitro drug release profile for plain emulsomes (mean ± S.D) (n=3). (B) In-vitro drug release profile for PAM -anchored emulsomes (mean ± S.D) (n=3).

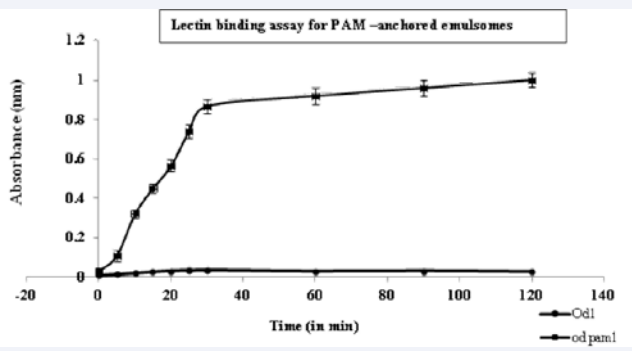

Figure 8 In vitro ligand specificity of PAM –anchored emulsomes by lectin induced aggregation method (mean ± S.D) (n=3).

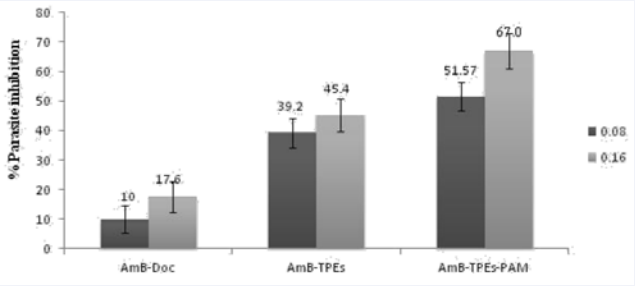

Figure 9 In vitro activity against intracellular amastigotes in macrophages (J774A.1) (mean ± S.D) (n=3).

24 hr of incubation from plain and PAM-anchored emulsomes (Results not shown), respectively. This may be attributed to the lipophilic nature of the drug as well as presence of solid lipid core, which was further stabilized by PC bilayers hence prevented the drug leaching in serum. Also, the PAM anchoring significantly reduced the leaching of the drug from emulsomes.

Effect of storage on size and percent residual drug content

Storage stability of both plain and PAM-anchored emulsomes was evaluated by measuring the change in particle size and percent residual drug content after storing them in tightly closed container at 37 ± 1ºC, 28 ± 1ºC and 4 ± 1°C for a period of 30 days. The increase in average particle size of emulsomes was found to be more upon storage at 37 ± 1°C and 28 ± 1°C as compared to 4 ± 1°C (Results not shown). This may be due to the fusion of the bilayer membranes of particles at higher temperature. The change in average particle size in the case of PAM-anchored emulsomes was relatively less as compared to plain emulsomes, which may be due to the hindrance to fusion imposed by PAM-anchoring on the emulsomes surface. Results for percent residual drug content indicate that 4 ± 1°C temperatures is suitable for their storage as after one month the residual drug content estimated was 95.2 ± 1.4 and 96.1 ± 2.4 % for plain and PAM -anchored emulsomes respectively. At 28 ± 1°C the % residual drug content after one month was estimated to be 93.2 ± 2.1 and 95.7 ± 2.2% for plain and PAM -anchored emulsomes respectively. However, at 37 ± 1ºC the % residual drug content after one month was estimated to be 92.6 ± 2.7 and 95.1 ± 2.7 for plain and PAM -anchored emulsomes respectively (data not shown). The data indicates that the changes in particle size and % residual drug content were not significant at the temperatures (4 ± 1°C, 28 ± 1°C and at 37 ± 1ºC). Therefore it is concluded that formulations are almost stable at 4 ± 1°C, 28 ± 1°C as well as 37 ± 1ºC.

In vivo serum and organ distribution studies

The organ distribution of the AmB bearing emulsomes (both plain and PAM anchored) was compared with the AmB-Doc (Table 3) in albino rats at a dose of 1 mg/kg. The biodistribution pattern studies clearly indicate that both plain (AmB-TPEs) and PAM anchored (AmB-TPEs-PAM) AmB emulsomes are superior as compared against the AmB-Doc in accumulating AmB in the macrophages rich organs like liver and spleen. The effect of these formulations was analyzed by using one way analysis of variance followed by post hoc Tukey's multiple comparison tests. AmB emulsomes (plain and PAM -anchored) showed a significant ($P < 0.05$) increase in the level of AmB in the macrophage rich organs (liver and spleen) as compared to free AmB.

The accumulation of free AmB in the liver and spleen was recorded to be 30.5 ± 0.97 and 10.56 ± 1.22%, respectively after 1 h and 2.88 ± 1.1 and 1.45 ± 1.33%, respectively after 24 h. Formulation AmB-TPEs deposited 54.38 ± 0.90 and 13.11 ± 1.34% of AmB after 1 h and 45.24 ± 0.89 and 10.87 ± 0.98% of AmB after 24 h in the liver and spleen, respectively. Formulation AmB-TPEs-PAM deposited 65.04 ± 0.70 and 51.70 ± 0.99% of AmB in liver and 18.29 ± 1.1 and 12.02 ± 1.1% in spleen after 1 h and 24 h, respectively (Table 3).

The intraperitoneal administration of free AmB (1 mg/kg body weight) resulted in relatively lower serum concentration of AmB (3.1 ± 0.89%) after 1 h which declined to 1.01 ± 0.91% as estimated at 10 h and which further declined to 0.4 ± 0.87 at 24 h. Serum concentrations of AmB after the administration of AmB-TPEs were significantly higher ($P < 0.05$) than the free drug, i.e. 4.82 ± 0.99 and 3.8 ± 1.34% after 1 and 10 h, respectively. PAM -anchored emulsomes (AmB-TPEs-PAM) exhibited still higher serum concentration (2.85 ± 0.84 and 2.1 ± 1.1% after 1 and 10 h, respectively) than free drug, but it was noticeably lower than that obtained after the administration of its plain counterpart (AmB-TPEs). The decrease in serum concentration in the case of PAM-anchored emulsomes was accompanied by a corresponding increase in drug accumulation in macrophage rich organs like liver, spleen and lung. The enhanced hepato-splenic and lung clearance of PAM -anchored emulsomes and entrapped AmB could be the probable reason of subsequent lower serum drug concentrations (Table 3).

Estimation of AmB accumulated in various organs reveals that AmB emulsomes significantly ($P<0.05$) alter the biodistribution pattern of the AmB-Doc. Although AmB-Doc itself accumulates in liver, spleen, lung and kidney, yet the rate, extent and duration of accumulations in the liver and spleen were significantly higher after the administration of AmB emulsomes ($P < 0.05$). The administration of the plain emulsome formulations exhibited higher rate and extent of passive accumulation of drug in the liver and spleen with a concomitant reduction in drug accumulation in kidney. This may be attributed to the colloidal nature of AmB emulsomes, which forced the lipid particles to accumulate passively in the macrophage rich organs. Anchoring of emulsomes with PAM further enhanced the accumulation of AmB selectively in liver (from 54.38 ± 0.90 to 65.4 ± 0.70%), spleen (from 13.11 ± 1.34 to 18.29 ± 1.1%) and lungs (from 4.05 ± 0.90 to 7.32± 0.7%) after 1 h as compared against plain emulsomes. The observed higher accumulation of these emulsomes as compared with their plain counterparts as well as the percent retention of AmB over the 24 h study period in macrophages of liver and spleen may be due to their greater and selective affinity for emulsomes coated with polysaccharide derivatives (e.g., amylopectin, palmitoyl conjugates of pullulan, pullulan phosphate) [5,11]. Comparison of the biodistribution patterns after the administration of PAM-anchored emulsomes suggests that anchored formulations exhibited higher accumulation levels in the liver and spleen as compared with their plain counterparts containing an equivalent

Table 3: Organ distribution of AmB following administration of control (free AmB, AmB-Doc) and emulsome entrapped AmB.

Formulation	Organs	% dose recovered			
		1 hrs	4 hrs	10 hrs	24 hrs
AmB-Doc	Serum	3.1 ± 0.89	1.65 ± 0.70	1.01 ± 0.91	0.4 ± 0.87
	Liver	30.5 ± 0.97	33.15 ± 0.88	18.5± 0.98	2.88± 1.1
	Spleen	10.56 ± 1.22	13.81 ± 1.1	8.4± 1.45	1.45 ± 1.33
	Lungs	2.9 ± 1.32	3.25 ± 1.79	1.09 ± 1.55	0.13 ± 1.44
	Kidney	3.98 ± 0.88	3.5 ± 1.1	1.23 ± 0.89	1.09 ± 0.97
AmB-TPE	Serum	4.82 ± 0.99	4.57 ± 1.22	3.8 ± 1.34	2.4 ± 1.5
(TPE3S4P4)	Liver	54.38 ± 0.90	59.8 ±0.89	52.4 ± 1.22	45.24 ± 0.89
	Spleen	13.11 ± 1.34	19.2 ± 1.5	14.1 ±0.84	10.87± 0.98
	Lungs	4.5 ± 0.90	5.11 ± 0.89	3.5 ± 1.22	1.48 ± 0.89
	Kidney	1.05 ± 1.22	4.52 ± 1.1	3.2 ± 1.45	0.75 ± 1.45
AmB-TPE-	Serum	2.85 ± 0.84	2.57 ± 0.98	2.1 ± 1.1	1.25 ± 1.56
PAM	Liver	65.4 ± 0.70	71.48 ± 0.91	56.44 ± 0.75	51.7 ± 0.99
(TPE3S4P4-	Spleen	18.29 ± 1.1	21.23 ± 1.56	16.4 ± 0.88	12.02 ± 1.1
PAM)	Lungs	7.32 ± 0.7	9.19 ± 0.91	4.2 ± 0.75	1.98 ± 0.99
	Kidney	0.95 ± 1.34	2.85 ± 1.5	1.1 ± 0.84	0.6 ± 0.89

Abbreviations: AmB: Amphotericin B; AmB-Doc: Amphotericin B deoxycholate

dose of AmB. Significant (P<0.05 in a rank sum test) statistical difference in the resulting biodistribution patterns excludes the probability of inter-/intra-subject variations substantiating the role of ligand–receptor interaction mediated phenomenon.

These findings help to conclude that considerably higher concentrations of drug could be maintained inside the macrophage rich organs over the protracted period of time. The observed values suggest that the ligand anchored emulsomes are not only effective in rapid attainment of high drug concentrations in macrophage rich organs but also maintain the concentration levels over a prolonged period of time, when compared against the free drug. This establishes the significance of the targeting potential of the developed systems [19].

In vivo antileishmanial activity testing

The antileishmanial activity of AmB (0.5 and 1 mg/kg) was investigated *in-vivo* against VL by the inhibition of parasitic load in the spleen of *L. donovani* infected hamsters after intraperitoneal injections of AmB-Doc (Mycol), formulation AmB-TPEs and AmB-TPEs-PAM. At the dose of 0.5 mg/kg, significant activity (P < 0.001 as compared to AmB-Doc) was observed using formulation AmB-TPEs-PAM causing 52.44 ± 3.9 % inhibition of splenic parasitic burden. Whereas, formulation AmB-TPEs and AmB-Doc caused only 32.86 ± 2.4 and 20.98 ± 2.10 % parasite inhibition respectively (P < 0.01 for AmB-TPEs vs. AmB-Doc) in leishmania infected hamsters. At the dose of 1 mg/kg, highly significant activity (P < 0.001 as compared to AmB-Doc) was observed using formulation AmB-TPEs-PAM causing 68.03 ± 4.2 % inhibition of splenic parasitic burden. Whereas, formulation AmB-TPEs and AmB-Doc caused only 39.66 ± 3.8 and 24.43 ± 3.55 % parasite inhibition respectively (P < 0.01 for AmB-TPEs vs. AmB-Doc) in leishmania infected hamsters (Figure 10A,10B). Empty TPEs as well as empty TPEs-PAM formulation also showed 9.52 ± 2.4 and

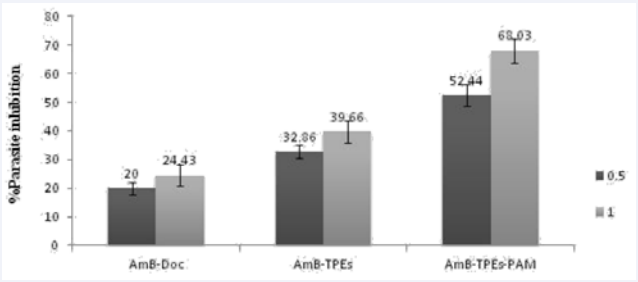

Figure 10a Antileishmanial activities of emulsomes against established infection of L. donovani in hamsters (mean ± S.D) (n=3).

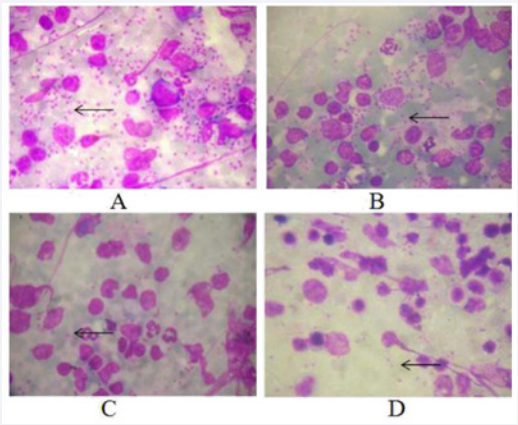

Figure 10b Photomicrographs showing giemsa stained splenic smears of Leishmania donovani infected hamsters from control and treated groups (x 100). A-untreated control group. B-AmB-Doc (AmB-Doc) treated group. C-Formulation TPEs treated group. D-Formulation TPEs-PAM treated group. Arrow (↓) indicates parasite.

12.67 ± 3.2 % parasite inhibitions respectively (data notshown in figure) which were significantly lower as compared to AmB-Doc ($P < 0.05$), TPES4P4 ($P < 0.001$) and TPES4P4-PAM ($P < 0.001$) (Figure 10A,10B).

Emulsomes have been recognized as potent drug delivery devices for the treatment of leishmaniasis because both emulsomes and leishmania parasite are taken up by the same reticuloendothelial system and it creates an ideal situation for a high degree of drug parasite interaction. Furthermore, if suitable ligands are covalently attached to emulsomes, so that they could easily be recognized by the macrophages (host for the parasite) receptors, then these customized emulsomes could probably be used successfully as carriers for site specific delivery [4,17]. Mannosylated liposome incorporated benzyl derivative of *Penicillium nigricans* derived compound MT81 has also been reported for the treatment of VL [15]. The majority of the drugs used formerly in the therapy of leishmaniasis were toxic. However when those drugs were liposome-encapsulated, they were found to be less toxic and more efficient in the therapy of leishmaniasis [20,21]. In hamsters the liposomal formulation of AmB given at a single dose of 11 mg/kg eliminated >99% of parasites, even in animals with a high parasite burden; in monkeys 4 mg/kg given in 3 days elicited >98% suppression of hepatosplenic parasites [22]. In our study the doses of 0.5 and 1 mg/kg given for 5 consecutive days were chosen for comparison of antileishmanial activity of emulsome encapsulated drug. Nevertheless, given the good tolerance of this formulation, higher doses are expected to be appropriate for achieving cure with total clearing of the parasites.

The efficacies of a series of AmB loaded carriers in the treatment of VL are reported [11,23,24]. In the present study the efficacy of AmB in the form of novel carrier, AmB-TPEs-PAM was tested against VL in hamsters and compared with AmB-Doc and AmB-TPEs. The formulation AmB-TPEs showed significant ($P < 0.01$) inhibition of splenic parasitic load as compared to AmB-Doc. This may be attributed to the colloidal nature of TPEs which forced the lipid particles to accumulate passively in the macrophage rich organs like spleen, hence related to a different drug biodistribution in the form of carrier. Formulation TPEs-PAM was found to be most potent in terms of inhibition of splenic parasitic load.

CONCLUSION

From the present study we conclude, that the approach of colloidal carriers was successful and the versatile carrier system i.e. emulsomes were proved to be effective for intracellular macrophage delivery of AmB. The formulations could significantly modify the pharmacokinetics of AmB, providing prolonged action at comparatively low drug doses thereby reducing the toxicity problems like nephrotoxicity, cardiac arrhythmia etc. The formulation of AmB in emulsomes (both plain and PAM-anchored) displays better antileishmanial activity as compared to AmB-Doc. The study also demonstrates that the efficacy of AmB against VL is increased if the drug is used in the PAM -anchored emulsomes. Furthermore, it shows that PAM -anchored emulsomes would probably have useful applications against other macrophage-associated disorders in the near future.

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REFERENCES

1. Singh N, Kaur J, Kumar P, Gupta S, Singh N, Ghosal A. An orally effective dihydropyrimidone (DHPM) analogue induces apoptosis like cell death in clinical isolates of *Leishmania donovani* overexpressing pteridine reductase 1. Parasitol. Res. 2009; 105:1317–1325.
2. Misra P, Kumar A, Khare P, Gupta S, Kumar N, Dube A. Pro-apoptotic effect of the landrace bangle mahoba of piper betla on leishmania donovani may be due to the high content of eugenol. J Med Microbiol. 2009; 58:1058–66.
3. Kunjachan S, Gupta S, Dwivedi AK, Dube A, Chourasia, M. Chitosan-based macrophage-mediated drug targeting for the treatment of experimental visceral leishmaniasis. J Microencapsul. 2011; 28: 301–310.
4. Pal A, Gupta S, Jaiswal A, Dube A, Vyas SP. Development and evaluation of tripalmitin emulsome for the treatment of experimental visceral leishmaniasis. J liposome Res. 2012; 22: 62–71.
5. Vyas SP, Quraishi S, Gupta S, Jaganathan KS. Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages. Int J Pharm. 2005; 296: 12– 25.
6. Nan A, Croft SL, Yardley V, Ghandehari H. Targetable water-soluble polymer-drug conjugates for the treatment of visceral leishmaniasis. J Control Release. 2004; 94:115–127.
7. Gupta S, Sharma P, Gupta MK. Leishmaniasis – drugs, nanotechnology based delivery systems and recent patents survey. Current Nanomedicine. 2016; 6: 2–42.
8. Ahsan F, Rivas IP, Khan MA, Torres SA. Targeting to macrophages: role of physicochemical properties of particulate carriers-liposomes and microspheres on the phagocytosis by macrophages. J Control Release. 2002; 79: 29–40.
9. Basu MK, Lala S. Macrophages specific drug delivery in experimental leishmaniasis. Curr Mol Med. 2004; 4: 681–689.
10. Gupta S, Dube A, Vyas SP. Development and characterization of amphotericin B loaded solid lipid nanoparticles against experimental visceral leishmaniasis. Pharmaceutical Nanotechnology. 2013; 1: 54-67.
11. Gupta S, Vyas SP. Development and characterization of amphotericin B bearing emulsomes for passive and active macrophage targeting. J Drug Target. 2007; 15: 206–217.
12. Khare P, Rastogi P, Gupta S, Maurya R, Dube A. *In vitro* and *in vivo* efficacy of a new herbaceous indian plant-*Abutilon indicum* against *Leishmania donovani* infection. Amer J Phytomed Clin Ther. 2014; 2: 134-139.
13. Sharma S, Kumar P, Jaiswal A, Dube A, Gupta S. Development and characterization of doxorubicin loaded microparticles against experimental visceral leishmaniasis. J Biomed Nanotech. 2011; 7: 135-136.
14. Vyas SP, Katare YK, Mishra V, Sihorkar V. Ligand directed macrophage targeting of amphotericin B loaded liposomes. Int J Pharm. 2000; 210:

1-14.

15. Mitra M, Mandal AK, Chatterjee TK, Das N. Targeting of mannosylated liposome incorporated benzyl derivative of Penicillium nigricans derived compound MT81 to reticuloendothelial systems for the treatment of visceral leishmaniasis. J drug target. 2005; 13 : 285-293.

16. Copland MJ, Baird MA, Rades T, McKenzie JL, Becker B, Reck F, et al. Liposomal delivery of antigen to human dendritic cells. Vaccine. 2003; 21: 883-890.

17. Gupta S, Dube A, Vyas SP. Antileishmanial efficacy of amphotericin B bearing emulsomes against experimental visceral leishmaniasis. J Drug Target. 2007; 15: 437-444.

18. Maupin KA, Liden D, Haab BB. The fine specificity of mannose-binding and galactose-binding lectins revealed using outlier motif analysis of glycan array data. Glycobiology. 2012; 22: 160-169.

19. Yadav S, Gupta S. Development and in vitro characterization of docetaxel loaded ligand appended solid fat nanoemulsions for potential use in breast cancer therapy. Artif Cells Nanomed Biotech. 2015; 43: 93-102.

20. Goldsmith DR, Perry CM. Amphotericin B lipid complex: In visceral leishmaniasis. Drugs. 2004; 64: 1905-1911.

21. Kafetzis DA, Velissariou IM, Stabouli S, Mavrikou M, Delis D, Liapi G. Treatment of paediatric visceral leishmaniasis: Amphotericin B or pentavalent antimony compounds. Int J Antimicrob Agents. 2005; 25: 26-30.

22. Berman JD, Hanson WL, Chapman WL, Alving CR, Lopez-Berestein G. Antileishmanial activity of liposome encapsulated amphotericin B in hamsters and monkeys. Antimicrob Agents Chemother. 1986; 30: 847-851.

23. Dea-Ayuela MA, Rama-Iniguez S, Sanchez-Brunete JA, Torrado JJ, Alunda JM, Bolas-Fernandez F. Anti-leishmanial activity of a new formulation of amphotericin B. Tropical Med Int Health. 2004; 9: 981-990.

24. Sánchez-Brunete JA, Dea MA, Rama S, Bolás F, Alunda JM, Raposo R, et al. Treatment of eaxperimental visceral leishmaniasis with amphotericin B in stable albumin microspheres. Antimicrob Agents Chemother. 2004; 48: 3246-3252.

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