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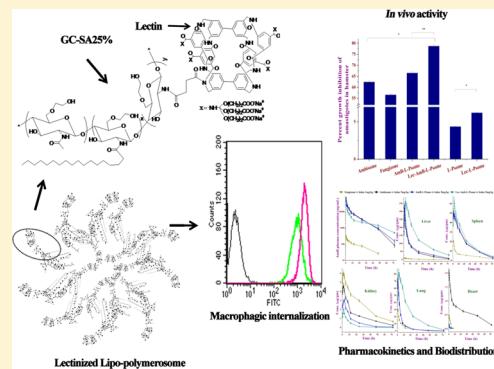
# Exploitation of Lectinized Lipo-Polymerosome Encapsulated Amphotericin B to Target Macrophages for Effective Chemotherapy of Visceral Leishmaniasis

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## S Supporting Information

**ABSTRACT:** We have designed lectin functionalized Lipo-polymerosome bearing Amphotericin B (Lec-AmB-L-Psome) for specific internalization via lectin receptors overexpressed on infected macrophages of mononuclear phagocytic system (MPS) for the effective management of intramacrophage diseases such as visceral leishmaniasis. The lipo-polymerosome composed of glycol chitosan-stearic acid copolymer (GC-SA<sub>25%</sub>) and model lipid cholesterol was surface-functionalized with lectin by the EDC/NHS carbodiimide coupling method. Our designed Lec-AmB-L-Psome showed >2-fold enhanced uptake and significantly higher internalization in macrophages as compared to AmB-L-Psome. Importantly, pharmacokinetic and organ distribution studies illustrate significantly higher accumulation of Lec-AmB-L-Psome in MPS especially in liver, spleen, and lung as compared to AmB-L-Psome, Ambisome, and Fungizone. The IC<sub>50</sub> value demonstrated that Lec-AmB-L-Psome has 1.63, 2.23, and 3.43 times higher activity than AmB-L-Psome ( $p < 0.01$ ), Ambisome ( $p < 0.05$ ), and Fungizone ( $p < 0.05$ ), respectively. Additionally, the Lec-AmB-L-Psome showed significantly higher splenic parasite inhibition ( $78.66 \pm 3.08\%$ ) compared to Fungizone and Ambisome that caused only  $56.54 \pm 3.91\%$  ( $p < 0.05$ ) and  $66.46 \pm 2.08\%$  ( $p < 0.05$ ) parasite inhibition, respectively, in *Leishmania*-infected hamsters. The toxicity profile revealed that Lec-AmB-L-Psome is a safe delivery system with diminished nephrotoxicity which is a limiting factor of Fungizone application. Taken together, these studies suggest that this surface functionalized self-assembled Lec-AmB-L-Psome can introduce a new platform to specifically target macrophages for effective management of intramacrophage diseases.



## INTRODUCTION

Visceral leishmaniasis (VL) is a derelict tropical infectious disease spread through infection with the trypanosomatid parasite *Leishmania donovani* that inhabits and proliferates inside the macrophages of the mononuclear phagocytic system (MPS) and is lethal if not treated adequately.

There are a number of limitations with the use of drugs available in the market including toxicity from the conventionally marketed formulation Fungizone and cost related issues from the lipid based formulation Ambisome.<sup>1</sup> To overcome these restrictions, drug targeting is essential for many drugs including Amphotericin B (AmB), which is not selective with regard to the cellular sites of uptake or action. More than ever, chemotherapy leads to action of the drug in nontarget tissues that causes unwanted side effects and then limits successful therapy.<sup>2</sup> Advancements in biotechnology, monoclonal antibodies,<sup>3</sup> and recombinant protein directed to specific sites were established as promising candidates for active targeting. Therefore, a specific ligand anchored delivery system (Lipo-polymerosome, L-Psome) was designed to meet the efficacy criteria that can target macrophages intracellularly without

toxicity to other cells and be cost-effective, which can open up new treatment avenues.

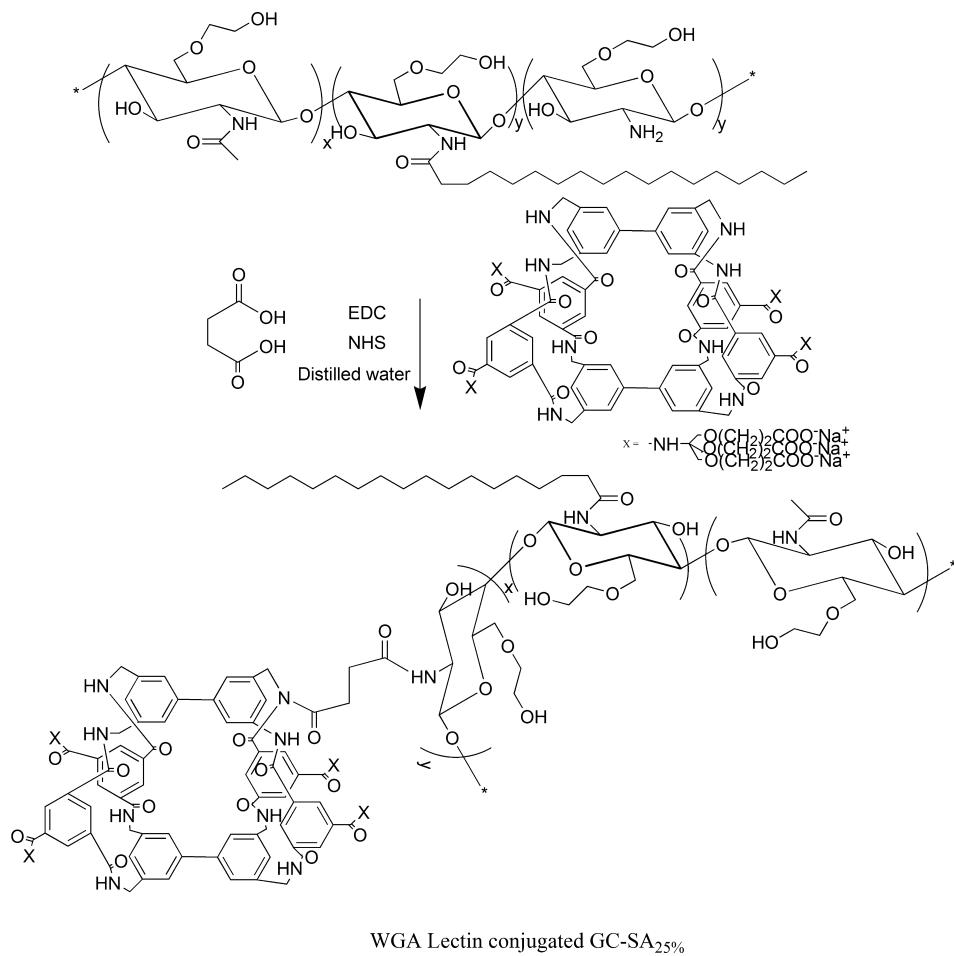
Lectins are actually proteins obtained from plant extracts and have been reported to have excellent bioadhesion properties through cell-cell adhesion in living beings.<sup>4</sup> Having carbohydrate specificity, the lectin is one of the potential molecules for drug targeting and can be exploited for site specific drug delivery. Wheat germ agglutinin (WGA), a type of lectin, is a carbohydrate free dimeric protein ( $M_w \sim 35$  kDa) consisting of two identical subunits each having two independent and identical N-acetyl glucosamine binding sites.<sup>4</sup> The oral toxicity of wheat germ is considered to be negligible, as this forms a part of the daily human diet, nearly 300 mg WGA/kg.<sup>5</sup>

It has also been reported that lectin binds to members of the LFA-1/H Mac-1/gp I 50,95 adhesion glycoprotein family on human neutrophils and macrophages;<sup>6</sup> CD18 monoclonal antibodies immunoprecipitated the  $\alpha L$ ,  $\alpha M$ ,  $\alpha X$ , and  $\beta$  chains

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**Figure 1.** Synthesis scheme of WGA lectin conjugation on GC-SA<sub>25%</sub>.

of this protein family, with the 135 kDa protein immunoprecipitated by CD15 antibodies from neutrophils and 183 kDa macrophage protein.<sup>7</sup> Lectin, present as terminal residues at the cell surface, contain siglecs which binds to sialic acid and N-glycolylneuraminic acid (Neu5Gc) binding sites.<sup>8</sup> The accessibility of these binding sites at the terminal position is considerably responsible for regulation of cellular and molecular interactions.<sup>9</sup> Neu5Gc is not present in healthy humans,<sup>10</sup> while it is present in leishmaniasis diseased human beings, making huge differences in Neu5Gc expression in *Leishmania* infected macrophage surfaces.<sup>8,11</sup>

The liver and macrophage membranes have lectin (basically N-acetyl glucosamine) receptors on their surfaces,<sup>12</sup> which identifies particular sugars on the surface of parasites and plays a vital role for the promastigote during infection in macrophages. Involvement of N-acetyl glucosamine receptors has been supported through the inhibition pattern exhibited by *in vitro* cellular attachment experiments. Furthermore, parasite clearance from blood was restrained only by neoglycoproteins and glycoproteins specific for N-acetyl glucosamine/mannose receptors.<sup>13</sup>

L-Psome composed of amphiphilic copolymers and lipid behaves similarly to vesicular drug carriers like liposomes and thus has the ability to deliver drug efficiently.<sup>14</sup> In the present investigation, the targeting potential of the lectin surface functionalized lipo-polymerosome bearing AmB (Lec-AmB-L-Psome) was explored and found that it has the ability to be taken up intracellularly with an improved pharmacokinetic and

pharmacodynamic profile. Together, the developed formulation has the potential for application in VL and intramacrophage diseases.

## RESULTS AND DISCUSSION

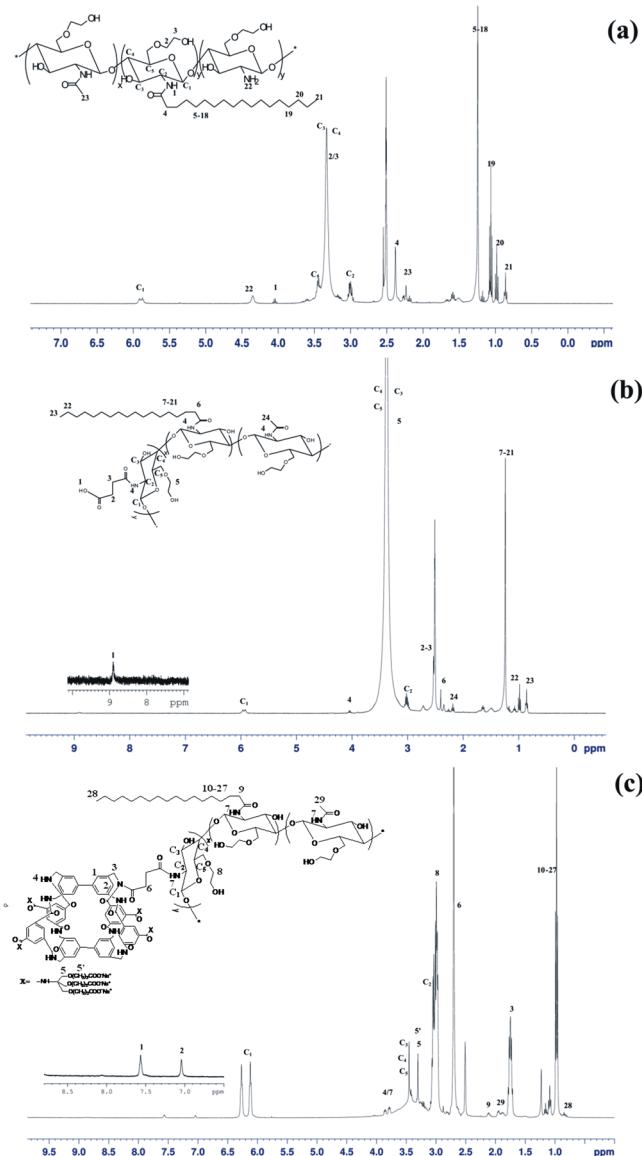
**Preparation and Characterization of AmB-L-Psome and Lec-AmB-L-Psome.** In this study, the prototype formulation was developed according to our previous report<sup>14</sup> using the amphiphilic copolymer of glycol chitosan (GC) as the hydrophilic shell, and stearic acid (SA) served as the hydrophobic core and cholesterol as the lipid that may be present at the interface of the GC shell and SA core by forming a lipid monolayer. We hypothesized that the surface functionalized lipo-polymerosome (Lec-L-Psome) with lectin combined the advantageous properties of liposomes and polymeric nanoparticles, eliminating few of their fundamental limitations such as drug payload, stability, rigidity, controlled and targeted release at the site of infection, solubility, and pharmacokinetic profile. The Lec-L-Psome is anticipated to contain three unique properties: (i) the hydrophobic domain of polymeric core which is biodegradable and has the ability to encapsulate lipophilic drugs/poorly water-soluble bioactive drug molecules and provide sustained drug release rate;<sup>14</sup> (ii) lipid present at the interface of the hydrophilic shell and hydrophobic core through monolayer formation adequately prevents free diffusion of drug from the L-Psome, and the hydrophilic shell of L-Psome restricts water penetration, which leads to enhanced stability and an increase in encapsulation efficiency

with sustained release properties of L-Psome,<sup>14</sup> and (iii) functionalization of the L-Psome surface is feasible with lectin/targeting ligands which may render unique binding properties to specific receptor or tissue.<sup>15</sup>

First, we prepared a copolymer of glycol chitosan-stearic acid (GC-SA<sub>25%</sub>) from GC and SA in molar ratio of 4:1 through the carbodiimide coupling reaction.<sup>14</sup> The prepared GC-SA<sub>25%</sub> copolymer and cholesterol were dissolved in DMAc and ethanol, respectively, which then self-assembled to minimize free energy on the concentric interfaces of water, so these self-assembled in aqueous phase to produce a core–shell structure by vigorous vortexing. After that, L-Psome was formed by the absolute removal of organic solvent through evaporation followed by sonication.<sup>14</sup> This technique represents an innovative, cost-effective, and preferable means for greater scalability of the self-assembled copolymer based L-Psome. Second, the lectin was conjugated on the L-Psome surface using a two-step EDC/NHS carbodiimide coupling method<sup>16</sup> with succinyl spacer between lectin and L-Psome as shown in Figure 1.

The reaction of lectin conjugated L-Psome was characterized through <sup>1</sup>H NMR spectroscopy (400 MHz, Bruker, Germany) and IR spectroscopy (PerkinElmer, Buckinghamshire, United Kingdom). The <sup>1</sup>H NMR spectrum of GC-SA<sub>25%</sub> (Figure 2) illustrates various peaks allied to protons attached with an anomeric proton C1 and other protons of C2, C3–C4, and C5 carbon atoms of the sugar, and methyl protons appeared at 5.91, 3.04, 3.26–3.37, 3.48, and 2.17 ppm, respectively. The –NH<sub>2</sub>, –NH, methylene, and characteristic methyl proton of stearic acid fall at 4.34, 4.08, 0.97–1.36, and 0.87 ppm, respectively. The NMR proton spectrum of intermediate succinyl-GC-SA<sub>25%</sub> (Figure 2) shows the characteristic peak of –COOH at 8.97 ppm and the –NH<sub>2</sub> peak at 4.34 ppm of GC-SA<sub>25%</sub> was diminished, and the methylene peak of succinyl appears at 2.58 ppm, while other peaks slightly shift from their original positions. In the final lectin conjugated GC-SA<sub>25%</sub>, compared to succinyl-GC-SA<sub>25%</sub> and GC-SA<sub>25%</sub>, the NMR proton spectrum (Figure 2) shows a diminishing carboxyl proton peak at 8.97 ppm and addition of two characteristic types of aromatic proton peaks due to conjugation of lectin on GC-SA<sub>25%</sub> at 7.08 and 7.59 ppm, as well as O-methylene and N-methylene peaks at 3.32 and 1.74 ppm, respectively; while the rest of the peaks in lectin conjugated GC-SA<sub>25%</sub> slightly shifted from their positions in succinyl-GC-SA<sub>25%</sub> and GC-SA<sub>25%</sub>. The ratio of integrals among one of the aromatic peaks of lectin and the anomeric proton peak of GC-SA<sub>25%</sub> at 6.21 ppm ensured quantification of the lectin percentage bound to the succinyl-GC-SA<sub>25%</sub> –COOH groups that was found to be 3.53%. These results confirm successful conjugation of Lec on the L-Psome surface.

The IR data of L-Psome (GC-SA<sub>25%</sub>) and Lec-L-Psome (Lec-succinyl-GC-SA<sub>25%</sub>) as shown in Figure S1 of the Supporting Information also confirms the conjugation of lectin on the L-Psome surface. The IR spectrum of GC-SA<sub>25%</sub> illustrates peaks at 1219.6, 1646.8, and 3425–3839 cm<sup>-1</sup> allied to –CH bending from GC–CO stretching (amide group among GC and SA), and –OH stretching vibrations (from GC) as shown in Supporting Information Figure S1(a). The IR peaks shift from 1646.8 to 1697.2 cm<sup>-1</sup> and from 1219.6 to 1297.5 cm<sup>-1</sup>, and introduction of a peak at 1818.2 cm<sup>-1</sup> (–CO stretching from carboxylic group of succinic acid) confirmed the formation of succinyl-GC-SA<sub>25%</sub> as shown in Supporting Information Figure S1(b). While lectin conjugation on succinyl-GC-SA<sub>25%</sub> was



**Figure 2.** <sup>1</sup>H NMR spectra of (a) GC-SA<sub>25%</sub> in DMSO-*d*<sub>6</sub>, (b) succinyl-GC-SA<sub>25%</sub> in DMSO-*d*<sub>6</sub>, and (c) lectin conjugated GC-SA<sub>25%</sub> in DMSO-*d*<sub>6</sub> solvent. Tetramethylsilane (TMS) used as internal standard in NMR spectroscopy.

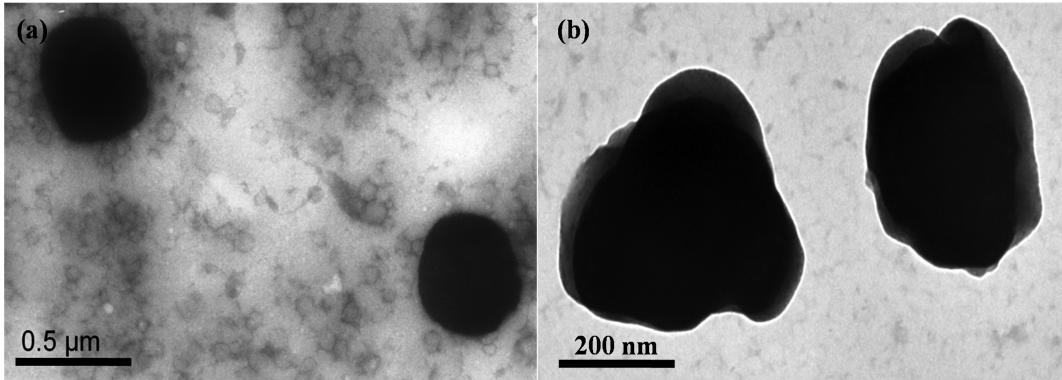
confirmed by disappearance of the IR peak 1818.2 cm<sup>-1</sup> and introduction of a peak at 1743.8 due to formation of an amide bond among the carboxyl group of succinyl and the amino group of lectin, however, other IR peaks 1697.2 cm<sup>-1</sup> slightly shift to 1682 cm<sup>-1</sup> (amide bond peak in between succinyl and GC-SA<sub>25%</sub>), 1651.5 cm<sup>-1</sup> (amide bond peak of GC-SA<sub>25%</sub>), and from 1297.5 to 1219 cm<sup>-1</sup> as shown in Supporting Information Figure S1(c).

The conjugation efficiency was measured by the BCA protein estimation method, for which unconjugated lectin was separated by Sephadex G100 silica gel column and subtracted from the initial amount of lectin. In this study, the conjugation efficiency was found to be  $5.20 \pm 0.40\%$  mg/mg for lectin with respect to L-Psome (Table 1). The conjugation efficiency measured by NANODROP 2000 was found to be  $5.01 \pm 0.32\%$  and  $4.98 \pm 0.57\%$  mg/mg lectin with respect to L-Psome for the Bradford and Lowry methods, respectively, as shown in Table 1. All the methods used in determination of conjugation

Table 1. Characterization of AmB Formulations<sup>a</sup>

name of formulation	particle size (nm)	polydispersity index (PDI)	zeta potential (mV)	drug loading (% w/w)	encapsulation efficiency (% w/w)	lectin bound (% w/w)		
						protein estimation method		
						BCA	Bradford	Lowry
AmB-L-Psome	341.1 ± 12.3	0.197 ± 0.02	(+) 19.32 ± 0.85	25.59 ± 0.87	89.58 ± 1.25	-	-	-
Lec-AmB-L-Psome	483.2 ± 25.1	0.241 ± 0.07	(+) 13.00 ± 0.34	25.37 ± 0.73	90.03 ± 1.78	5.20 ± 0.40	5.01 ± 0.32	4.98 ± 0.57

<sup>a</sup>Each data point represents the mean ± standard deviation ( $n = 3$ ).

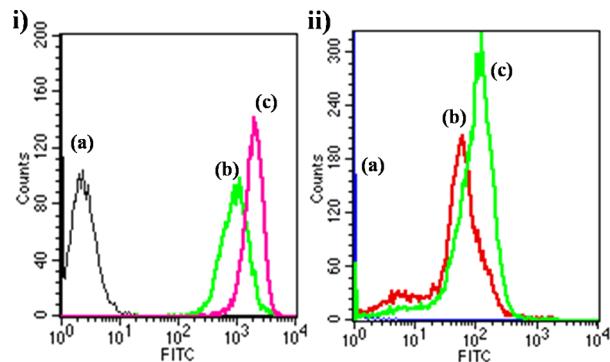


**Figure 3.** HRTEM (high resolution transmission electron microscopy) microphotographs of (a) optimized AmB-L-Psome and (b) optimized Lec-AmB-L-Psome. Lectin conjugation was observed as a layering over AmB-L-Psome.

efficiency were not significantly different for lectin conjugation on L-Psome.

Figure 3a,b shows the morphological characteristics of the L-Psome and Lec-L-Psome by HRTEM. Lectin conjugation was depicted as a layering over L-Psome as shown in Figure 3b. Entrapment efficiency in L-Psome was found to be 89.58 ± 1.25% w/w; however, no significant change was observed on lectin conjugation as shown in Table 1. Initially, zeta potential of L-Psome was found to be (+) 19.32 ± 0.85 mV due to GC, with a mean diameter of 341.1 ± 12.3 nm (polydispersity index (PDI), 0.197 ± 0.02). Lectin conjugation on L-Psome decreases its positive zeta-potential of (+) 13.00 ± 0.34 mV due to neutralization of some of the positive charge of amino groups on GC and increase in the mean diameter of 483.2 ± 25.1 nm (PDI, 0.241 ± 0.07) as shown in Table 1. Increase in size and decrease in zeta potential due to neutralization of some of the positive charge of the L-Psome from the Malvern Zetasizer suggests conjugation of lectin on the L-Psome.

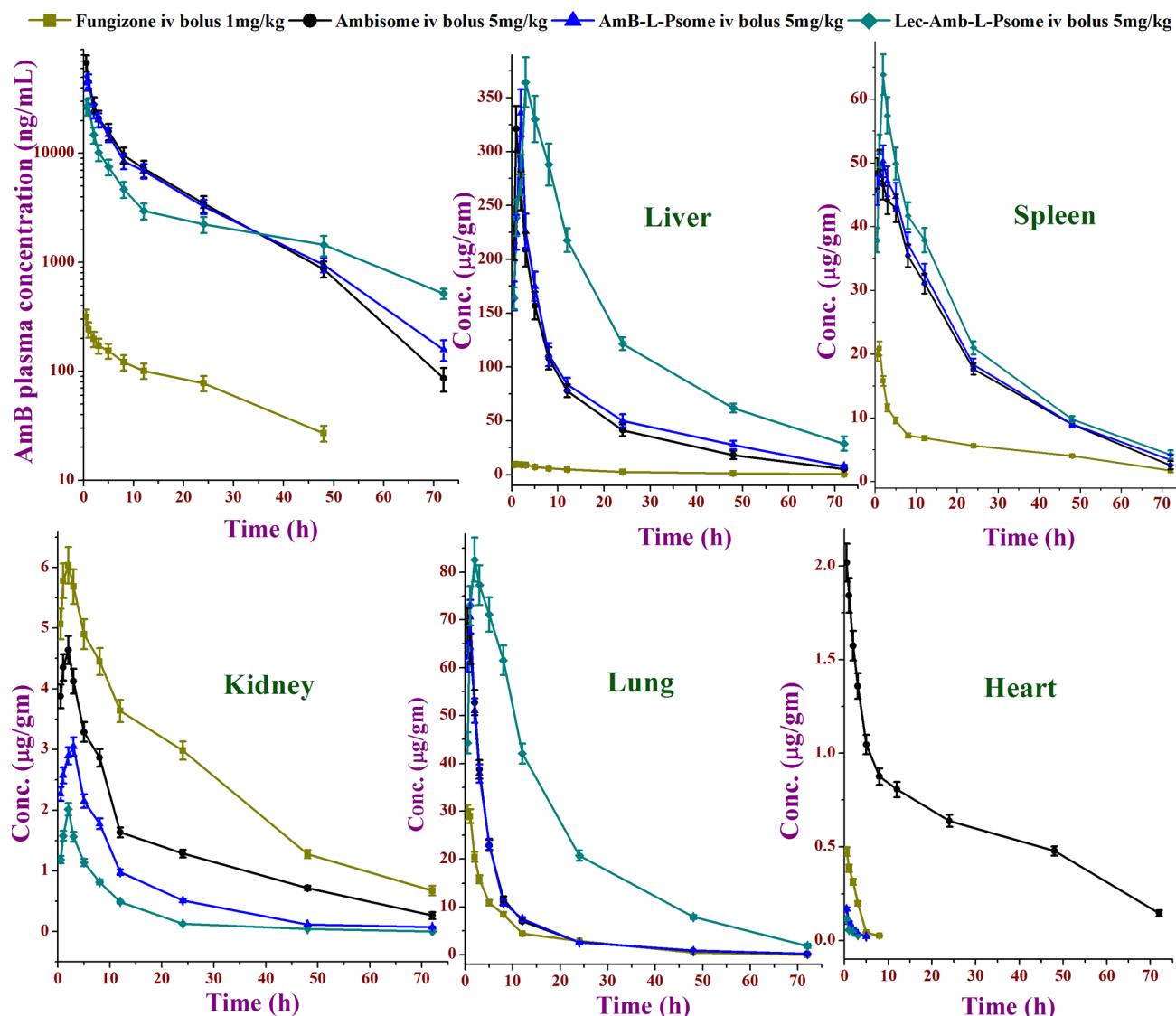
**In Vitro Uptake Study.** When observed through TLC, the RF value was slightly higher for FITC-AmB than AmB, while it was lower than FITC, which indicates that the polarity of free AmB is very near that of FITC-AmB. This observation provides evidence that partitioning of both tagged and free AmB remains enclosed in L-Psome and Lec-L-Psome. The degree of cellular uptake was quantified by calculating the mean fluorescence value obtained by differently tagged FITC formulations as shown in Figure 4. In the relative concentrations of FITC tagged formulations Lec-AmB-L-Psome, AmB-L-Psome, and control, the macrophage uptake was significantly higher (~2-fold) and (~2.3-fold) in J774A and RAW264.7 macrophagic cell lines, respectively. In the case of tagged Lec-AmB-L-Psome, mean fluorescence value was 1750.19 and 139.02 FL1-H, while it was 890.37 and 59.03 FL1-H in the case of AmB-L-Psome, whereas the control group showed mean fluorescence value



**Figure 4.** In vitro uptake study on (i) J774A macrophage cell line after 6 h incubation of (a) control, (b) FITC tagged AmB-L-Psome, and (c) FITC tagged Lec-AmB-L-Psome; (ii) RAW264.7 macrophage cell line after 6 h incubation of (a) control, (b) FITC tagged AmB-L-Psome, and (c) FITC tagged Lec-AmB-L-Psome.

2.57 and 1 FL1-H as shown in Figure 4. This higher uptake of Lec-AmB-L-Psome may be attributed to lectin conjugation which facilitates biahesion to the cell membrane.

This data supports our hypothesis of superior uptake of lectin conjugated L-Psome all the way through N-acetyl glucosamine lectin receptors widely overexpressed on macrophages and hepatocytes.<sup>12</sup> This higher uptake of Lec-AmB-L-Psome compared with that of AmB-L-Psome in macrophages provides potential indications for targeting intramacrophage amastigotes in spleen and liver using lectin modified L-Psome for chemotherapy of VL. The higher phagocytic potentiation values by lectin might be attributed to a higher number of N-acetyl glucosamine binding sites of the lectin present on the macrophages. It may thus be concluded that the surface architecture of macrophages, projecting several sugars, plays a pivotal role in forming bridges among formulations by serving



**Figure 5.** Plasma AmB concentration–time semilogarithmic plot (mean  $\pm$  SEM); liver, spleen, lung, kidney, and heart AmB concentration–time plot (mean  $\pm$  SEM) following i.v. bolus administration of Fungizone 1 mg/kg, Ambisome 5 mg/kg, AmB-L-Psome 5 mg/kg, and Lec-AmB-L-Psome 5 mg/kg ( $n = 3$ ).

**Table 2. Pharmacokinetic Parameters of AmB Formulations<sup>a</sup>**

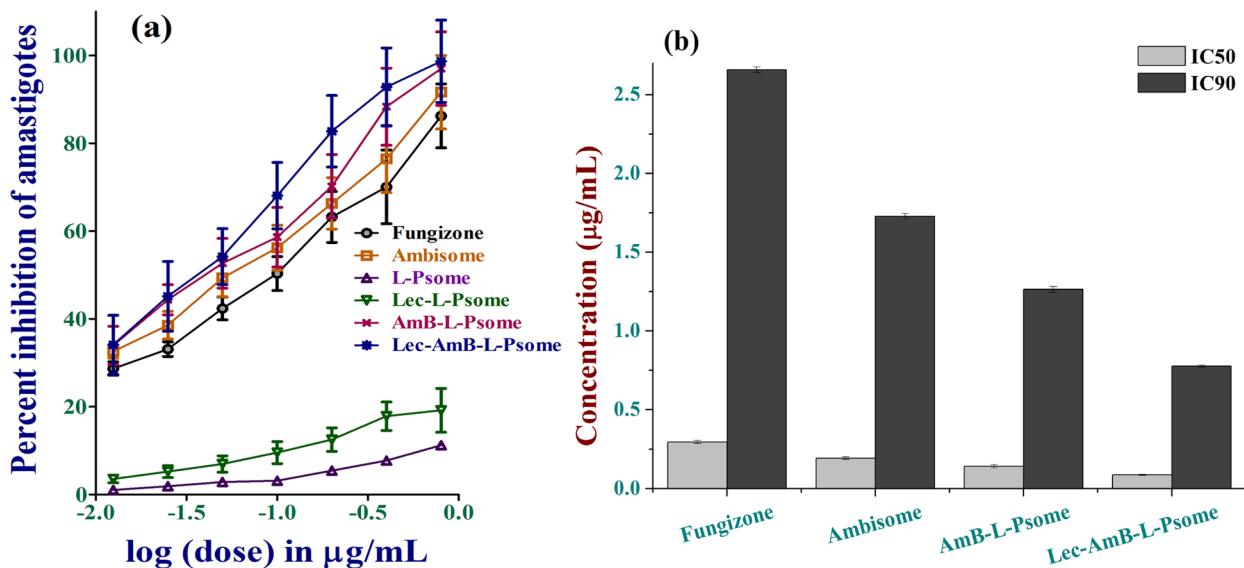
AmB equivalent concentration in formulation	Fungizone 1 mg/kg	Ambisome 5 mg/kg	AmB-L-Psome 5 mg/kg	Lec-AmB-L-Psome 5 mg/kg
$AUC_{0-72h}$ (h $\times$ ng/mL)	$4464.4 \pm 526.3$	$342739.3 \pm 28461.5$	$317360.3 \pm 48697$	$204075.4 \pm 21648$
$t_{1/2}$ (h)	$17.7 \pm 2.6$	$11.8 \pm 1.9$	$12.6 \pm 3.4$	$55.6 \pm 8.9$
CL (mL/h/kg)	$207.0 \pm 21.8$	$14.5 \pm 1.6$	$15.6 \pm 2.5$	$22.6 \pm 3.7$
MRT (h)	$15.9 \pm 4.1$	$11.5 \pm 2.8$	$12.9 \pm 3.1$	$24.7 \pm 5.2$

<sup>a</sup>Each data represents the mean  $\pm$  standard deviation ( $n = 3$ ).

as receptors for phagocytosis.<sup>17</sup> N-Acetyl glucosamine lectin receptors are widely overexpressed on MPS, and our formulation was surface modified with lectin that might have interacted in two different ways: (i) Multivalent lectin present on the surface directly interacts with four sugar binding sites of lectin receptors present on the macrophages of host tissues leading to vacuolation and subsequently internalization.<sup>18</sup> This phenomenon has been supported by our data as shown in Figure 4, showing internalization of the lectin conjugated L-Psome by the macrophage cells. (ii) Cytoinvasion of lectin increases absorption of the formulation from macrophages

because of high specificity and high binding capacity.<sup>19</sup> The intrinsic properties of lectin, i.e., cytoadhesion and cytoinvasion, remained unchanged after successful conjugation on the surface of L-Psome. Indeed, it has been reported that coadministration of free lectin reduces uptake of formulation (data not shown) through inhibition of lectin-mediated endocytosis.<sup>16</sup> The significantly higher internalization creates awareness to facilitate an innovative scope for enhanced localization of drug in the reticuloendothelial system as shown in Figure 4.

#### Pharmacokinetic Profile and Tissue Distribution of AmB-Formulations Following IV Bolus Administration.



**Figure 6.** (a) In vitro dose–response curves of Lec-AmB-L-Psome, AmB-L-Psome, Ambisome, Fungizone, Lec-L-psome, and L-Psome with different concentrations of formulations against *L. donovani* amastigote infected macrophages observed after 48 h of incubation ( $n = 3$ ). (b) In vitro anti-leishmanial activity ( $\text{IC}_{50}$  and  $\text{IC}_{90}$ ) of Lec-AmB-L-Psome, AmB-L-Psome, Fungizone, and Ambisome in *L. donovani* amastigote infected macrophages observed after 48 h of incubation ( $n = 3$ ).

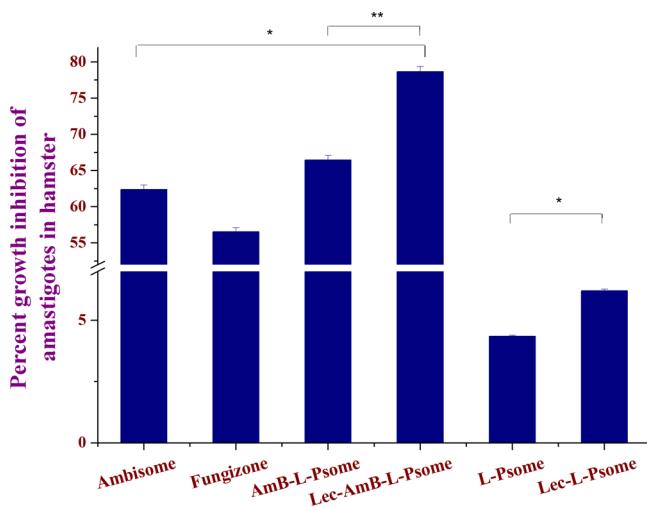
To understand the superior uptake of Lec-AmB-L-Psome, we have compared tissue distribution (1 g each of spleen, liver, lung, heart, and kidney tissue) and pharmacokinetics of AmB after intravenous administration of both marketed formulations and Lec-AmB-L-Psome. As shown in Figure 5 and Table 2, Lec-AmB-L-Psome and AmB-L-Psome showed lower plasma drug concentrations than Ambisome. This observation could be attributed to sustained release rendered by enhanced rigidity and molecular interaction with GC-SA<sub>25%</sub>,<sup>14</sup> which may delay release of AmB. Initially after 30 min of administration, the concentration of Lec-AmB-L-Psome was lower as compared to that of Ambisome and AmB-L-Psome; however, the trough concentration of AmB was higher even after 72 h of Lec-AmB-L-Psome administration. This may be attributed to saturation kinetics leading to redistribution of AmB in to the systemic circulation. Pharmacokinetic results show nonlinear saturation-like kinetics of Lec-AmB-L-Psome resulting in sustained plasma levels and increased mean residence time (MRT) and half-life. The AUC of Lec-AmB-L-Psome will be significantly underestimated based on the plasma profile in Figure 5. It is because of the highly specific macrophage targeting ligand, lectin anchored on the surface of AmB-L-Psome, which directs the restricted distribution of Lec-AmB-L-Psome toward liver, spleen, and lung. This complex pharmacokinetics possibly promotes pharmacotherapy using AmB wherein there is a reduction in possible toxicity by avoiding undue exposure to normal cells/tissues.

The Lec-AmB-L-Psome, being composed of macrophagic targeting ligand and lectin conjugated on the AmB-L-Psome surface, is recognized more rapidly through MPS, as liver, lung, spleen, and so forth cause rapid clearance at the initial phase from the bloodstream and result in significantly lower plasma concentration compared to Ambisome and AmB-L-Psome, as shown in Figure 5. The result of tissue distribution of AmB formulations at different time points following i.v. administration is shown in Figure 5. In tissues, the AUC and MRT of Lec-AmB-L-Psome were higher compared to that of Ambisome and AmB-L-Psome, with a corresponding decrease in clearance

as shown in Figure 5. The data shows that AmB remains in tissues for a prolonged period and slowly “seeps out” back into systemic circulation following slow elimination from the body. The prolonged storage of AmB in tissues is justified by multicompartmental analysis of results and previous reports.<sup>20</sup> The AmB becomes more concentrated in the liver when administered with Lec-AmB-L-Psome due to active targeting through lectin receptors present at the surface of liver macrophages, while Ambisome showed relatively lower concentration (Figure 5). The Lec-AmB-L-Psome shows higher disposition in spleen compared to AmB-L-Psome and Ambisome due to its physicochemical properties. In addition, Lec-AmB-L-Psome shows preferential distribution into lungs due to the presence of lectin receptors and very low distribution in kidney, whereas distribution of Ambisome among lung, heart, and kidney was nearly equal. All formulations of AmB (except Ambisome) demonstrated very low distribution in the heart tissues. Since Fungizone is a micellar formulation of AmB, it gets distributed similarly among liver, lung, spleen, kidney, and heart tissues after administration as shown in Figure 5. Fungizone has favored disposition in the kidney tissue, though it becomes obvious and leads to nephrotoxicity caused by Fungizone than lipid based formulations of AmB.<sup>39</sup> It has been reported that different components, i.e., Poloxamer 188 and/or poly(caprolactone) of new formulations of AmB, significantly modify the biodistribution profile of AmB in the body.<sup>21</sup> However, we have incorporated highly specific lectin receptor binding ligands on the surface of L-Psome that have provided a better pharmacokinetic and biodistribution profile compared to marketed formulations and are highly specific for infected target organs such as liver, spleen, and lung.

**Inhibitory Effect on *L. donovani* Intramacrophage Amastigotes and in Vivo Anti-Leishmanial Activity.** The in vitro inhibitory effect of different formulations on *L. donovani* intramacrophage amastigotes has been shown through a dose–response curve and  $\text{IC}_{50}$ , as well as  $\text{IC}_{90}$  in Figure 6. This demonstrates that the activity of Lec-AmB-L-Psome formula-

tion is 1.63, 2.23, and 3.43 times higher compared to that of AmB-L-Psome ( $p < 0.01$ ), Ambisome ( $p < 0.05$ ), and Fungizone ( $p < 0.05$ ), respectively. The in vivo experimental results as shown in Figure 7 clearly indicate that Lec-AmB-L-



**Figure 7.** In vivo anti-leishmanial activity of Lec-AmB-L-Psome, AmB-L-Psome, Fungizone, and Ambisome in the established Syrian golden hamster model infected with *L. donovani* amastigotes at an intraperitoneal dose of 1 mg of AmB/kg body weight of hamster and formulations without drug were injected intraperitoneally into each hamster on day 31 post-infection. The parasite burden was estimated by splenic biopsy on day 8 post-treatment and percentage parasite inhibition was calculated in comparison with the parasite burden of untreated infected animals (means  $\pm$  SD) ( $n = 5$ ). The mean parasite burden in the spleen of untreated, infected control animals was  $454 \pm 38$  amastigotes per 100 cell nuclei of macrophages ( $n = 5$ ). \*  $p < 0.05$  (comparison of Lec-AmB-L-Psome versus Fungizone and Ambisome, Lec-L-Psome versus L-Psome), \*\*  $p < 0.01$  (comparison of Lec-AmB-L-Psome versus AmB-L-Psome).

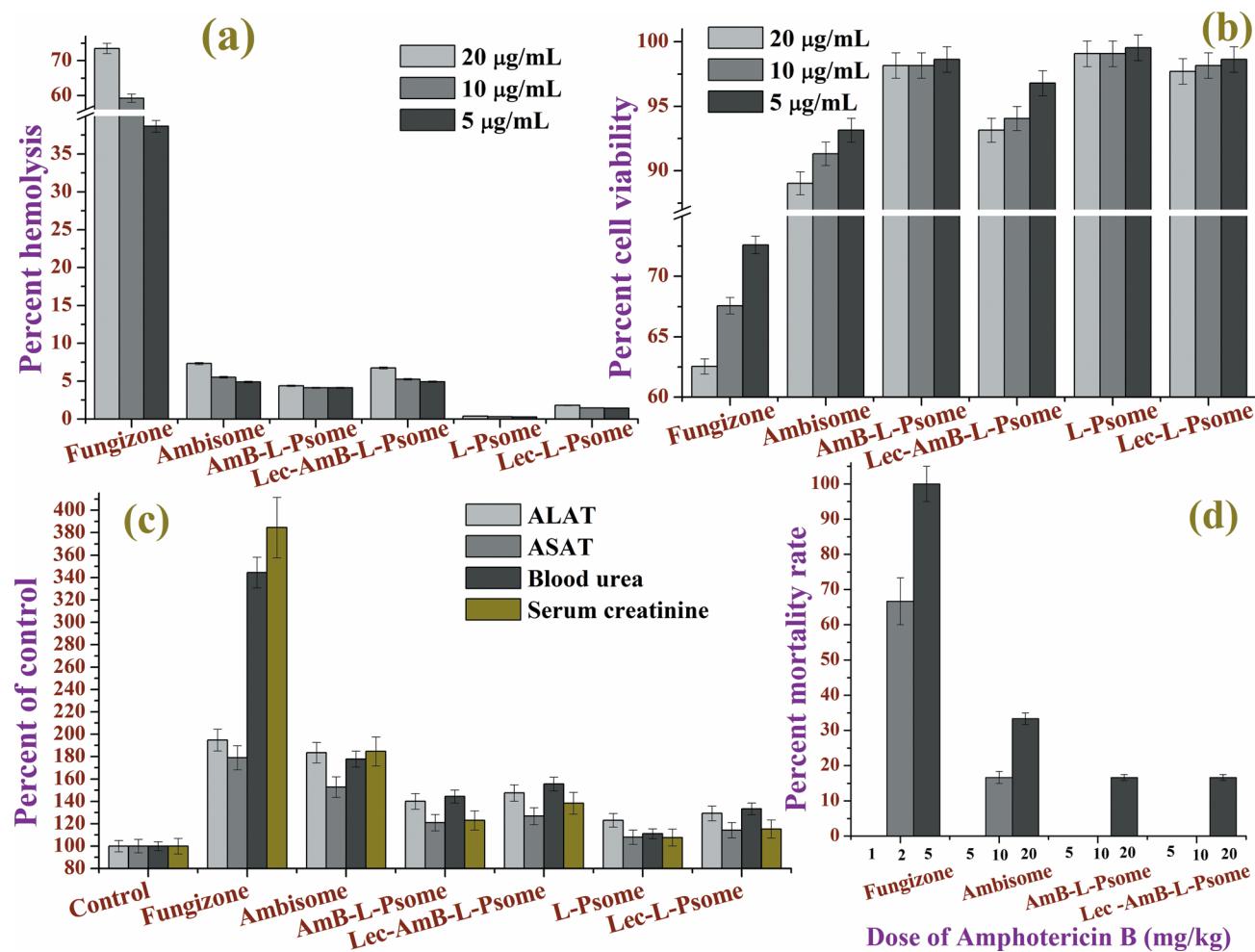
Psome was significantly more active ( $78.66 \pm 3.08\%$  inhibition) in contrast to Fungizone ( $56.54 \pm 3.91\%$  inhibition) ( $p < 0.05$ ), whereas inhibition shown by Ambisome and AmB-L-Psome was  $62.37 \pm 3.13\%$  ( $p < 0.05$ ) and  $66.46 \pm 2.08\%$  ( $p < 0.01$ ), respectively.

The anti-leishmanial activity of Lec-AmB-L-Psome both in vitro and in vivo was significantly improved in contrast to that of marketed formulations, i.e., Ambisome and Fungizone ( $p < 0.05$ ), since the macrophagic targeting potential of lectin conjugated L-Psome advanced a new area of targeted drug delivery systems for leishmaniasis chemotherapy. To the best of our knowledge, we are the first to explore the targeting potential of lectin in L-Psome, a drug delivery system for leishmaniasis. Our data also illustrate that Lec-L-Psome can be a potential delivery system for targeting anti-leishmanial drugs inside macrophages, and importantly, Lec-L-Psome, itself has shown anti-leishmanial activity. In view of the fact that lectin conjugated L-Psome proceeds as an indication for engulfment via phagocytes which are specifically recognized by macrophages and ultimately phagocytosed, consequently resulting in activation of macrophages.<sup>22</sup> It has also been reported that up-regulation of cytokines and inducible nitric oxide synthase (iNOS) (data not shown) and successive production of nitric oxide are also key issues for the parasitcidal activity in infected macrophages throughout experimental infections.<sup>36</sup> Polymer GC has been shown to induce a pro-inflammatory response and

subsequently activate macrophages that results in digestion and quick parasite killing.<sup>23,24</sup> The WGA type of lectin also activates macrophages by lectin-dependent macrophage-mediated cytotoxic reaction in which the 170 kDa and 110 kDa proteins of the macrophage cell surface play an important role in which WGA can bind and activate macrophages.<sup>25</sup> Hence, enhanced drug uptake and effective killing of *L. donovani* parasites from the Lec-AmB-L-Psome formulation are anticipated to achieve the desired therapeutic effect at a lower dose. However, for parenteral administration of drug a small dose regimen is suitable for patients and also reduces side effects.

**Toxicity Assay of the Formulations.** Figure 8a,b showed that Fungizone has very high hemolysis and cell cytotoxicity on Wistar rat RBCs and J744A cell lines, respectively, even at low doses due to the presence of multimeric and aggregated forms of drug present in the formulation, while Lec-AmB-L-Psome, AmB-L-Psome, and Ambisome are relatively less toxic owing to the monomeric form of the drug.<sup>14</sup> This observation complies with the previous report wherein high toxicity of AmB in human erythrocytes and other cultured mammalian cells has been reported due to the aggregated form compared to the monomeric form of the drug, as reported earlier.<sup>26</sup> The Fungizone and lipid-based formulation Ambisome showed higher levels of serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) after biochemical analysis, which leads to a marked increase in the hepatotoxicity (Figure 8c); these results are consistent with an earlier report.<sup>27</sup> This might be because of proportion and composition of the lipid allied with AmB. However, copolymer–lipid based AmB-L-Psome and Lec-AmB-L-Psome did not increase ASAT and ALAT levels significantly after administration in mice (Figure 8c). The serum creatinine and blood urea levels significantly increase more than 3-fold ( $p < 0.05$ ) in the case of Fungizone over the control, while Ambisome, AmB-L-Psome, and Lec-AmB-L-psome had relatively similar results in renal function parameters as shown in Figure 8c. Serum creatinine and blood urea values revealed nephrotoxicity in mice following administration of Fungizone, but diminished it in others. Acute toxicity results suggest that only 16.66% mortality was found in mice at doses up to 20 mg/kg for Lec-AmB-L-Psome and AmB-L-Psome, while 33.33% mortality was observed with Ambisome at same dose as shown in Figure 8d. Mice injected with Lec-AmB-L-psome and AmB-L-Psome showed higher tolerance to AmB as revealed by tolerated dose (20 mg/kg) of AmB in mice as compared to Fungizone, which causes 100% mortality at the dose of 5 mg/kg as shown in Figure 8d and prevented further escalation of the dose in the case of Fungizone. No apparent nephrotoxicity was observed with Lec-AmB-L-Psome even at higher doses, and it was found to be safe. This may be due to the presence of the monomeric form of AmB in the formulation, and AmB was not freely available for normal cells/tissues. Moreover, lectin incorporation did not add further toxicity as compared to lipid-complexed AmB formulations.<sup>5,28,29</sup> The magnitude of cytokine induction remains below the threshold level (data not shown); even then it is sufficient to kill parasites without imposing any toxic side effects.<sup>30</sup> Similarly we have not observed any toxicity during our studies.

Furthermore, the excipients used in the development of Lec-AmB-L-Psome are relatively cost-effective and the process therefore is simple and reproducible as compared to the commercial formulation Ambisome.<sup>31</sup> With this unique



**Figure 8.** Effect of Fungizone, Ambisome, AmB-L-Psome, Lec-AmB-L-Psome, Lec-L-Psome, and L-Psome equivalent to Amphotericin B concentrations of 5, 10, and 20 µg/mL on (a) RBCs collected from Wistar rat blood; (b) J774A macrophage cell line ( $n = 3$ ). Serum biochemical analysis of (c) transaminases: aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT). Serum creatinine and blood urea of mice received saline (control group), Fungizone (1 mg/kg), Ambisome (5 mg/kg), Lec-AmB-L-Psome (5 mg/kg), AmB-L-Psome (5 mg/kg), Lec-L-Psome, and L-Psome intravenously in a constant volume of 200 µL daily for 15 days ( $n = 3$ ). (d) Percent mortality rate of 12 mouse group ( $n = 6$ ) treated with Fungizone (1, 2, 5 mg/kg), Ambisome (5, 10, 20 mg/kg), Lec-AmB-L-Psome (5, 10, 20 mg/kg), and AmB-L-Psome (5, 10, 20 mg/kg) through the intraperitoneal route.

advantage the application of this formulation developed for visceral leishmaniasis may soon be ready.

## CONCLUSION

The Lec-AmB-L-Psome is novel and simple to prepare with great scalability. The different receptor mediated uptake of formulations and biodistribution–pharmacokinetic profile significantly affect the efficacy and toxicity. The results of our experiments point out the relationship between the uptake of AmB and its efficacy, toxicity, and distribution. The Lec-AmB-L-Psome and AmB-L-Psome formulations have more improved therapeutic index than the commercial formulation Fungizone due to elimination of their toxicity, with increased efficacy. Finally, our formulation Lec-AmB-L-Psome significantly improves chemotherapy of intramacrophage infections, i.e., visceral leishmaniasis.

## MATERIALS AND METHODS

**Polymer and Chemicals.** Glycol chitosan (GC,  $M_w$  90 kD, degree of deacetylation 82.7%), [1-ethyl-3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC), stearic acid (SA),

cholesterol, fluorescein isothiocyanate (FITC), phosphotungstic acid, potassium dihydrogen phosphate, Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI) 1640, streptomycin, fetal bovine serum (FBS), wheat germ agglutinin (WGA), a type of lectin from *Triticum vulgaris*, and dimethylacetamide (DMAc) were purchased from Sigma-Aldrich (St. Louis, MO). Amphotericin B (AmB) was procured from Emcure pharmaceutical Ltd. (Pune, India) as a gift sample. The rest of the chemicals utilized in the experiments were of analytical grade and used as received. In all the experiments, required water was taken by Milli-Q Plus 185 water purification system.

**Preparation of AmB Encapsulated Lipo-Polymerosome (AmB-L-Psome).** A nanoprecipitation method was utilized for preparation of L-Psome through self-assembly followed by sonication as described in our previous work.<sup>14</sup> First, we prepared a copolymer of glycol chitosan-stearic acid (GC-SA<sub>25%</sub>) from GC and SA in a molar ratio of 4:1 through carbodiimide coupling reaction. The completely unmodified GC was separated by the dialysis method with the help of dialysis membrane tubing (MWCO ~100 kDa). Second, 4 mg

of prepared GC-SA<sub>25%</sub> copolymer, 1 mg of AmB in 100  $\mu$ L of DMAc, and 1 mg of cholesterol were taken in 250  $\mu$ L of ethanol and mixed gradually. This lipid solution was then gradually added using a syringe in 1 mL of distilled water under constant stirring for 5 min in 5 mL vial. The resultant dispersion was processed for size reduction by sonication for 120 s using a probe sonicator (Misonix, Germany) at 20% amplitude (15 s pulse on and 5 s pulse off), followed by dialysis with the help of dialysis membrane tubing (MWCO ~12000) against 1000 mL water for 2 h in order to remove unentrapped AmB and solvents.

**Preparation of Lectin Conjugated L-Psome.** For preparation of lectin conjugated L-Psome (Lec-L-Psome), a two step carbodiimide coupling method was used with few modifications.<sup>32</sup> Briefly, 4 mg of succinic acid (1 mol equiv) with 8 mg of EDC (1.2 mol equiv) was solubilized in 1 mL distilled water to activate the succinic acid. This activated succinic acid was added dropwise in a L-Psome suspension (5 mg/mL) and held with magnetic stirring for 12 h at RT. After that, 8 mg of EDC (1.2 mol equiv) and 8 mg of NHS (1.2 mol equiv) were added in the reaction mixture, followed by pouring of 1 mL lectin solution made in PBS (1 mg/mL) and stirred continuously for 12 h. The final product was separated by sephadex column (Sephadex G100) and unbound lectin and other molecules were eluted later. The experiment was repeated many times to estimate reproducibility of method.

**Characterization of AmB-L-Psome and Lec-AmB-L-Psome.** The L-Psome and Lec-L-Psome were analyzed for size and morphology using high resolution transmission electron microscopy (HRTEM, Tecnai G<sup>2</sup> F20, Eindhoven, The Netherlands) in distilled water. For this, 3 mM forman (0.5% plastic powder in amyl acetate) coated copper grid (300 mesh) negatively stained by 1% phosphotungstic acid was used at 200 kV. The Zetasizer (Nano ZS, Malvern Instruments, UK) was used for determination of average mean value of zeta potential and particle size through the dynamic light scattering technique in appropriate dilution with distilled water. The conjugation of lectin on L-Psome surface was also identified by <sup>1</sup>H NMR spectroscopy (400 MHz, Bruker, Germany) and IR spectroscopy (PerkinElmer, Buckinghamshire, United Kingdom).

To quantify lectin anchoring to L-Psome, the BCA protein estimation method was used.<sup>33</sup> In order to obtain bound lectin on L-Psome, the amount of unbound lectin eluted from a Sephadax G100 silica gel column was estimated using the ELISA microplate reader and subtracted from the initial amount used. To confirm lectin conjugation on L-Psome, we have also used the Bradford and Lowry methods for direct estimation of protein, after eluting conjugated lectin from a Sephadax G100 silica gel column, and then measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).<sup>34,35</sup> The coupling efficiency was calculated using the following formulas as the percentage of lectin bound to L-Psome from its initial amount:

$$\begin{aligned} \text{\% conjugation efficiency} \\ = \text{wt of (initial - unconjugated) or conjugated lectin} \\ \times 100/\text{total wt of L-Psome} \end{aligned}$$

The solvent injection method was used to encapsulate AmB in Lec-L-Psome, for which AmB was dissolved in DMAc and subsequently added in Lec-L-Psome with constant stirring. The amount of AmB entrapped in Lec-L-Psome was calculated by

analyzing the amount recovered of free AmB from the supernatant using reverse phase HPLC<sup>36</sup> after ultracentrifugation (40 000g for 30 min) and successive washings of the Lec-L-Psome formulation. The encapsulation efficiency was expressed as the percentage of drug entrapped in Lec-L-Psome compared to initially added drug.

**Tagging of AmB with FITC and Encapsulation of FITC Tagged AmB in Lec-L-Psome, L-Psome.** To understand the internalization/uptake of our formulations from macrophages, the AmB was converted to a fluorescent nature by tagging with fluorescent dye FITC using the previously reported method with slight modifications.<sup>37</sup> In brief, 10 mg of AmB and 5 mg of FITC were dissolved in 2 mL DMAc in the presence of triethylamine (200  $\mu$ L) in a 5 mL round-bottom flask and held with constant stirring at room temperature for 2 h. After that, to precipitate the product, 10 mL of ethyl acetate was poured in the reaction mixture and it was separated by centrifugation (18 000g for 10 min), then dried in vacuum over a desiccant. AmB, FITC, and product were identified by running TLC in a mobile phase consisting of ethyl acetate:methanol in 2:3 ratio. To encapsulate FITC tagged AmB in the L-Psome and Lec-L-Psome, FITC tagged AmB (1 mg) was dissolved in DMAc (100  $\mu$ L) and added dropwise into blank L-Psome and Lec-L-Psome using the solvent injection method. Free tagged drug was separated from the bulk of self-assembled tagged drug encapsulated L-Psome and Lec-L-Psome by centrifugation and washing.

**Cell Culture.** J774A and RAW264.7 macrophage cells were obtained from the animal cell culture unit (CDRI, Lucknow, India). Macrophage cells were cultured in DMEM supplemented with 10% FBS, 1% antibiotic, and antimycotic agent (Sigma, USA) at 37 °C in a humidified 5% CO<sub>2</sub> air atmosphere.<sup>36</sup>

**In Vitro Uptake Study.** For macrophage uptake assay, 12-well plates were seeded separately with J774A and RAW264.7 macrophage cells at a density of  $2 \times 10^5$  cells per well along with 800  $\mu$ L of complete DMEM. Twenty-four hours post incubation, the culture medium was replenished with fresh medium. FITC tagged formulations (AmB-L-Psome and Lec-AmB-L-Psome) at 10  $\mu$ g/mL of equivalent AmB concentration were placed in well plates and incubated for 6 h in humidified 5% CO<sub>2</sub> air atmosphere at 37 °C. After incubation, macrophage cells were washed twice, transferred in vials, and relative green fluorescence intensity was recorded with a FACS Calibur (Becton Dickinson, Oxford, UK) at  $\lambda_{\text{EX}}$  (495 nm) and  $\lambda_{\text{EM}}$  (525 nm).

**In Vitro Activity against Intramacrophage Amastigote in *L. donovani*.** The activity of test and commercial AmB formulations against intramacrophage amastigotes was assessed using the protocol reported previously.<sup>36</sup> In brief, J774A macrophages ( $10^5$  cells/well) growing in 24 well plates (Nunc, IL, USA) were infected with metacyclic green fluorescent protein (GFP) expressing promastigotes at 10:1 (parasite/macrophage) ratio and incubated at 37 °C in humidified 5% CO<sub>2</sub> air atmosphere for 12 h. Non-phagocytosed promastigotes were removed by washing thrice with PBS (pH 7.2) and the wells were resupplemented with complete medium. The cells were treated with AmB formulations (Lec-AmB-L-Psome, AmB-L-Psome, Fungizone, and Ambisome) at different drug concentrations, as well as with drug free Lec-L-Psome and L-Psome, with the equivalent amount of formulations and incubated for 48 h. Thereafter, cells were removed, centrifuged, washed two times in PBS, and transferred to tubes, and

subsequently fluorescence intensity was recorded by FACS calibur equipped with a 20 mW argon laser at  $\lambda_{\text{EX}}$  (488 nm) and  $\lambda_{\text{EM}}$  (515 nm), and data were analyzed using Kaluza flow cytometry analysis software (Beckman Coulter). The non-treated infected macrophages served as control. The in vitro anti-leishmanial activity of formulations was estimated by calculating the growth inhibition levels of the parasite through relative fluorescence of drug-treated parasites to nontreated control parasites. Each assessment was performed in triplicate.

**Animals.** Five- to six-week old Wistar male rats ( $205 \pm 8$  g, for pharmacokinetic analysis), male Syrian golden hamsters ( $45 \pm 4$  g, for in vivo anti-leishmanial activity), and female Swiss mice ( $25 \pm 3$  g, for acute toxicity) were made available by the National Laboratory Animal Center (CSIR-CDRI, Lucknow, India). The experimental animals were bred and reared in sterile conditions at  $24^\circ\text{C}$  and fed sterilized food and water (National Laboratory Animal Center, CDRI, Lucknow, India). All animal experimental procedures were in compliance with the guidelines for laboratory animal care and practice approved by the Indian Animal Ethics Committee (IAEC, IAEC approval no. CDRI/2012/38) and according to regulations of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India.

**In Vivo Studies. In Vivo Assessment of Anti-Leishmanial Activity.** The in vivo anti-leishmanial potential of test and commercial AmB formulations was investigated at an equivalent dose of 1 mg AmB/kg body weight toward *L. donovani* amastigotes in the Golden hamster.<sup>14,36</sup> In short, pelleted cultures of *L. donovani* promastigotes (5–6-day-old stationary phase) were taken and washed two times in PBS and resuspended in 1 mL PBS. For infection, hamsters were intracardially inoculated with immediately prepared resuspended parasites ( $1 \times 10^7$  promastigotes per 100  $\mu\text{L}$  of PBS). Thirty days after infection, the infected hamsters ( $n = 5$  in each group) were intraperitoneally administered AmB encapsulated formulations at a dose of 1 mg AmB/kg body weight per day for five successive days, the control group involved infected untreated hamsters. One week post treatment, animal groups were sacrificed, and their splenic aspirate smear was fixed with methanol, stained with Giemsa, and examined under a microscope to count the number of amastigotes per 100 nucleated macrophages. The mean parasite load in splenic smear of untreated infected animal group was  $454 \pm 38$  amastigotes per 100 macrophage nuclei ( $n = 5$ ). The experiment was repeated three times and the percentage of inhibition (PI) of amastigotes was calculated by using the following formula:<sup>38</sup>

$$\text{PI} = (\text{PP} - \text{PT}/\text{PP}) \times 100$$

where PP is the number of amastigotes per 100 macrophage nuclei in spleen of control group and PT is the number of amastigotes per 100 macrophage nuclei in spleen of drug-treated animal group.

**Pharmacokinetic and Biodistribution Study. Analytical Procedure.** To quantify AmB, analysis of plasma and tissue samples was carried out according to our published validated isocratic RP-HPLC method with slight modifications,<sup>36</sup> using a Shimadzu gradient liquid chromatographic system (model LC-10A series, Shimadzu, Japan) consisting of a 10 ATVP pump, Rheodyne injector (20  $\mu\text{L}$  loop) (7125 model; CA, US), AVP UV detector (SPD-M10; Shimadzu, Japan), and column [LichroCART 250–4, Lichrospher 100, RP-18e, 5  $\mu\text{m}$ , 250  $\times$

4 mm (Merck KgaA, 64271 Darmstadt, Germany)] with the help of the mobile phase [acetonitrile/10 mM KH<sub>2</sub>PO<sub>4</sub> buffer, at pH 4 (60:40, v/v)] at a constant flow rate of 1 mL/min. Homogenization of tissues (3–4 min) was carried out in acetonitrile (1 g of tissue/2 mL of acetonitrile) using IKA T25 digital ULTRA-TURRAX in an ice bath. The plasma and homogenized tissue samples in aliquots of 20  $\mu\text{L}$  were analyzed by RP-HPLC. The samples obtained from plasma and tissues show a linear calibration curve for AmB in the range of 1–10  $\mu\text{g}/\text{mL}$  and the limit of quantification are 20 ng/mL and 20 ng/g, respectively.

**Experimental Design.** The animals were divided into four groups and administered i.v. bolus injections of 5 mg/kg AmB-L-Psome ( $n = 6$ ), 5 mg/kg Lec-AmB-L-Psome ( $n = 6$ ), 1 mg/kg Fungizone ( $n = 6$ ), and 5 mg/kg Ambisome ( $n = 6$ ); where injection was given slowly to avoid the chance of a acute toxic reaction to AmB. In the present study, the range of i.v. doses of commercial AmB formulations was selected to be devoid of clinical nephrotoxicity according to previously published reports.<sup>39</sup> Blood samples (0.25 mL) were collected at 10 min predose and 0.5, 1, 2, 3, 5, 8, 12, 24, 48, and 72 h postdose of AmB formulation administration; and replaced with the same volume of normal saline to avoid hypovolemia. Collected samples were centrifuged (2500g, 10 min, 15 °C) to separate plasma and stored at  $-80^\circ\text{C}$  until drug analysis. The experimental mice were sacrificed at 0.5, 1, 2, 3, 5, 8, 12, 24, 48, and 72 h post administration of AmB formulations, and liver, spleen, right lung, right kidney, and heart were collected and kept at  $-80^\circ\text{C}$  until drug analysis.

**Toxicity Assay of the Formulations.** The in vitro hemolysis and J774A macrophage toxicity experiment was executed for different formulations (Lec-AmB-L-Psome, AmB-L-Psome, Fungizone, and Ambisome) at two AmB concentration levels (equivalent to 5 to 20  $\mu\text{g}/\text{mL}$ ). The equivalent drug free blank L-Psome and Lec-L-Psome were also tested according to previously reported methods.<sup>36</sup>

A subacute toxicity experiment was executed in seven mice groups having three animals in each, intravenously administered for 15 successive days with Lec-AmB-L-Psome (5 mg/kg), AmB-L-Psome (5 mg/kg), Ambisome (5 mg/kg), Fungizone (1 mg/kg), Lec-L-Psome, L-Psome, and saline (control group) in 200  $\mu\text{L}$  constant volume. Twelve hours post treatment of the last dose, treated animals were euthanized and blood samples were obtained by cardiac puncture. The samples were centrifuged (2000g) and subsequently analyzed for biochemical parameters (transaminases, urea, and creatinine) using an automatic Hitachi 912 apparatus (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Acute toxicity was studied through intraperitoneal injection in increasing doses of the Lec-AmB-L-Psome (5–20 mg/kg), AmB-L-Psome (5–20 mg/kg), Fungizone (1–5 mg/kg), and Ambisome (5–20 mg/kg), to different mice groups ( $n = 6$ ). Following administration, mortality of the animals was monitored for the next 8 h:

$$\% \text{ mortality rate} = (\text{number of dead mice}/\text{total number of mice}) \times 100$$

**Statistical Analysis.** All the analysis results are presented as mean  $\pm$  SD of three independent measurements. Data were compared using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test by

using statistical Graph Pad Prism 6 software (Graph Pad Software Inc., CA, USA).

## ■ ASSOCIATED CONTENT

### Supporting Information

IR spectra and interpretation of glycol chitosan-stearic acid (GC-SA<sub>25%</sub>) copolymer, succinylated glycol chitosan stearic acid, and lectin functionalized glycol chitosan-stearic acid. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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### Notes

The authors declare no competing financial interest.

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