

Research Paper

Amphotericin B-Gum Arabic Conjugates: Synthesis, Toxicity, Bioavailability, and Activities Against *Leishmania* and Fungi

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Purpose. Gum arabic, a branched polysaccharide consisting of more than 90% arabinogalactan having a molecular weight around 250,000 Da is the oldest and best known of all natural gums. The objective of the present investigation was to examine whether amphotericin B (AmB), the polyene antibiotic when conjugated to periodate oxidized gum arabic still retained its anti-fungal and anti-leishmanial activity and to evaluate its toxicity and bioavailability.

Methods. AmB conjugated to the oxidized polysaccharide through Schiff's linkages in the unreduced (imine) and reduced (amine) forms were characterized for the drug content, hemolytic potential, molecular mass, *in vitro* release and were examined for anti-fungal activity against *Candida albicans* and *Cryptococcus neoformans* and for anti-leishmanial activity against promastigotes of *Leishmania donovani* in culture. Toxicity and bioavailability were evaluated by intravenous (i.v) injections of the conjugates in mice and rabbits respectively.

Results. The conjugates were found to be non-hemolytic and mice withstood a dosage of 20 mg (AmB)/kg body weight of both conjugates. Histological examination of the internal organs of mice showed no lesions in kidney, brain, heart or liver. Estimation of the residual drug in the internal organs 7 days post injection showed that the spleen still retained 8.4±0.53 µg/g of tissue. AmB was found to be released from both conjugates *in vitro* although the release from the imine conjugate was much faster than from the amine conjugate. The concentrations inhibiting parasite growth by 50% (IC₅₀) values for the imine conjugate against promastigotes of *L. donovani* LV9 and DD8 strains were 0.37±0.04 and 1.44±0.18 µM respectively. The IC₅₀ values for the amine conjugates were much higher. The minimum inhibitory concentration (MIC) against *C. albicans* and *C. neoformans* was in the range of 0.5–0.9 µg/mL for both imino and amino conjugates. The bioavailability of the conjugate in rabbits showed that the imine conjugate maintained a plasma concentration in the range of 20 to 5 µg/mL while for the amine conjugate it was in the range of 17 to 3 µg/mL over 24 h.

Conclusions. The drug conjugates were stable, non-hemolytic and non-toxic to the internal organs of the animal and showed good anti-fungal and anti-leishmanial activity *in vitro*. In spite of the large molecular weight of the polysaccharide, AmB from the conjugates showed bioavailability after i.v injection. Since the highest concentration of AmB was found in the spleen after a single injection, these conjugates may have potential in anti-leishmanial therapy.

KEY WORDS: amphotericin B; antifungal; antileishmanial; bioavailability; gum arabic; hemolysis; polymer-drug conjugate; toxicity.

A. J. dedicates this paper to Prof. M. S. Valiathan for his 72nd birthday.

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INTRODUCTION

Life threatening opportunistic fungal infections are major causes of morbidity and mortality, especially in immunocompromised patients (1,2). Amphotericin B (AmB) is the drug of choice for the treatment of systemic fungal infections (3). AmB is also widely used as a second-line treatment for visceral and mucocutaneous leishmaniasis (4). The use of AmB is limited due to dose related side effects such as nausea, fever and chills and toxicity especially nephrotoxicity (5,6).

A major draw back of AmB is that it is insoluble in water and most organic solvents. It must be solubilized in an aqueous milieu to make it biologically active. The manner in which that is done, as well as the route by which it is administered determine its biological activity (7). Several strategies including chemical and physical modifications have

been examined to reduce the toxicity of AmB. The physical modification of AmB has resulted in a number of successful formulations. The formulation available in market, Fungizone[®] is a colloidal dispersion of AmB in sodium deoxycholate. Fungizone[®] however, has acute adverse events related to infusion toxicity and is often ineffective in immunocompromised patients (8). Liposomal AmB formulations are also available in market (AmBisome[®], Amphotec[™], Abelcet[®]) and are considerably less toxic compared to Fungizone[®] (9–11). However, infusion related side effects have been noticed for all these formulations at high doses. The high cost of production, the instability of liposomes and the necessity for continuous i.v. infusions however, prevent their widespread use.

A number of chemical modifications have also been attempted on AmB. Thus, Domb et al. conjugated AmB to a water-soluble polysaccharide arabinogalactan, which is reported to overcome many limitations of AmB such as its insolubility and toxicity (12–15). These conjugates were found to be more effective than both liposomal and deoxycholate formulations in eradicating yeast cells from target organs and were also effective and safe against leishmanial parasite infections. AmB has been covalently conjugated to ergosterol (16), and poly(vinyl pyrrolidone) (17) and the resulting conjugates were found to be biologically active.

Gum arabic is the oldest and best known of all natural gums. It is a highly branched, water soluble natural polysaccharide obtained from the exudates of acacia tree and is extensively used in food, pharma and cosmetic industry. Molecular structure of gum arabic consists mainly of three components: the major component being arabinogalactan (> 90%) with a low (0.5%) protein content, the second being arabinogalactan (< 10%) with a high protein content (10%) and the third component consisting less than 1% includes glycoprotein having around 50% protein content (18). It has a molecular weight about ten times that of arabinogalactan. It is considerably less expensive compared to arabinogalactan and is available in plenty. This study was therefore undertaken in order to examine whether AmB conjugated to a higher molecular weight polysaccharide would still show its antileishmanial and antifungal activity combined with suitable bioavailability with a low toxicity.

MATERIALS AND METHODS

Materials

Gum arabic of approximate molecular weight 250,000 (Product No. G-9752), Amphotericin B, dimethyl sulfoxide (DMSO), dimethylthiazol diphenyltetrazolium bromide (MTT), borax, boric acid, bovine serum albumin (BSA) and sodium *m*-periodate were purchased from Sigma, U.S.A. Dialysis tubing (Spectra/Por) was from Spectrum Laboratories Inc., CA., USA. Polysaccharide standards ($M_w/M_n < 1.2$) for molecular weight determination were from Polymer Laboratories, Amherst, MA, USA. All other reagents such as disodium hydrogen phosphate, monosodium hydrogen phosphate, sodium borohydride, dextrose, trisodium citrate, citric acid, morpholine propane sulfonic acid etc. were of analytical grade and were procured locally. Doubly distilled

water was employed throughout. HEPES, RPMI 1640 and fetal calf serum were from Life Technologies, Cergy-Pontoise, France. Gentamycin was from Schering-Plough (Levallois-Perret, France). Two clones of *Leishmania donovani* promastigotes were used in the study: *L. donovani* (MHOM/ET/L82/LV9) currently called LV9 from Africa and *L. donovani* (MHOM/IN/80/DD8) currently called DD8 from India. *Candida albicans* (ATCC 18804) and *Cryptococcus neoformans* (ATCC 84873) were obtained from American Type Culture Collection, Rockville, MD, USA. All the animal experiments were performed with the approval of the SCTIMST animal ethics committee.

Methods

Oxidation of Gum Arabic

Gum arabic was oxidized using sodium *m*-periodate as reported earlier (19). Briefly, into 100 mL of a 10% solution of gum arabic (0.058 mol) in distilled water was introduced 2.5 g of periodate (0.0116 mol) to obtain 20% oxidization and the contents were stirred magnetically at 20°C in the dark for 6 h. The extent of oxidation was determined iodometrically at the end of 6 h. After the reaction, contents were dialyzed (MWCO 6000–8000) against distilled water for 48 h with several changes of water until the dialysate was free from periodate. The solution was then frozen and lyophilized to dryness and stored in the dessicator at 4°C until use. Typical yield ranged 75–80%.

Molecular Weight Measurements

The weight average molar mass (M_w) of gum arabic and oxidized gum arabic was determined by gel permeation chromatography (GPC) using a Waters HPLC system equipped with 510 pump, R401 refractive index detector and 7725 Rheodyne injector. The column used was Ultra-hydrogel 1000/500/250. The mobile phase was 0.1 M NaNO₃ at a flow rate of 1 mL/min. A standard curve was prepared with retention time against $\ln M_w$ using polysaccharides of known molecular weight, and from the standard curve, M_w of the sample was determined.

Synthesis of Amphotericin B-Gum Arabic Conjugates

The synthesis was carried out according to Domb et al. (13). In a typical experiment, into a 1% solution of oxidized gum arabic in borate buffer (0.1 M, pH 11), adequate amount of AmB was added to obtain a drug payload of 20 wt.% and stirred continuously in dark for 48 h at 40°C. The imine conjugate thus obtained was purified by dialysis (MWCO 6000–8000) against distilled water (3 L) with several changes of water over 48 h and lyophilized to dryness. The imine conjugate was reduced with sodium borohydride to produce more stable amine conjugate. Sodium borohydride was added to the reaction mixture of imine conjugate (1.2 mol NaBH₄ / mol of saccharide unit in the polymer) with overnight stirring at room temperature. The amine conjugate thus formed was purified by dialysis in a similar fashion and lyophilized. The extent of AmB conjugated to gum arabic was estimated by UV absorption at 405 nm (Spectronic Genesys 2, Milton Roy,

NY) with a calibration curve of conjugates (12). The imine and amine conjugates showed a drug payload of 19.08 ± 1.7 and 19.33 ± 0.49 wt.% respectively.

In Vitro AmB Release Studies

In vitro release of AmB from the conjugates was examined in phosphate buffered saline (PBS, 0.1 M, pH 7.4). Drug release studies were conducted in a 2-compartment Perspex diffusion cell separated by a dialysis membrane of MWCO 3500 having area of diffusion of 12 cm². AmB conjugates, 50 mg was taken in 5 mL PBS in one compartment and the other compartment was filled with 5 mL PBS. The diffusion cell was incubated at 37°C and at definite intervals of time, the PBS was removed, replenished with 5 mL fresh PBS and AmB released was estimated by UV absorbance at 405 nm. To measure the release into albumin solution, the study was conducted in a similar fashion using 0.9 mM bovine serum albumin solution instead of PBS.

Hemolysis

Hemolytic potential of gum arabic-AmB conjugates was determined using erythrocytes isolated from human blood according to Larabi et al. (11). A stock solution of AmB was prepared in DMSO by dissolving 10 mg AmB in 10 mL of the solvent. It was diluted further with PBS to give concentrations in the range of 2–10 µg/mL. AmB conjugates were dissolved in PBS at different concentrations (equivalent to 200–1,000 µg AmB/mL). After incubation for 5 min at 37°C, erythrocytes were added to a final hematocrit of 2% and incubated for 30 min at 37°C. After centrifugation (1,500xg for 5 min at 4°C), the supernatant was removed and the erythrocyte pellet was lysed with sterile distilled water. The hemoglobin (Hb) remaining in the pellet was estimated from its absorption at 560 nm. Erythrocytes incubated in PBS alone served as the control to estimate total Hb content after lysis. The percentage hemolysis was calculated from the difference between the Hb remaining in the test samples and that remaining in the control erythrocytes. Experiments were done in triplicate.

Toxicity in Animal Model

Male albino BALB/c mice weighing about 20 g were injected through the tail vein with various doses (5, 20, 35, 50, and 100 mg AmB/kg body weight) of AmB-gum arabic conjugates in 0.5 mL of 5% dextrose. Tail vein injection volume of 0.5 mL was necessary in order to administer the maximum AmB dose of 100 mg/kg body weight which corresponded to 10 mg of the conjugate in 20 g mice. No hydrodynamic disorder was observed after the injection. Each group of mice (ten animals in each group) received one treatment of a specific dose of AmB formulation. Control mice received 0.5 mL of 5% dextrose. The conjugate was sterilized with ethylene oxide using standard protocols before dissolution in dextrose. Mice were monitored for 7 days. The group of mice that received 20 mg/kg body weight AmB was sacrificed on the eighth day. Their

internal organs liver, kidney, spleen, and lungs were homogenized, extracted with methanol, concentrated and examined for AmB by HPLC (Waters, equipped with model 510 pump, Restek C 18 column and 486 tunable absorbance detector). Injection volume was 100 µL at a flow rate of 1 mL/min. The mobile phase was acetonitrile/ water/ 0.05 M ammonium acetate buffer (40/14.5/45.5) and AmB was detected at 405 nm (15).

Histopathology

Histopathological examination of internal organs extracted from the test mice that received AmB-gum arabic imine conjugate and control mice was carried out. The internal organs were fixed in formalin, paraffin embedded and sections of 3–5 µm stained with hematoxylin and eosin and examined.

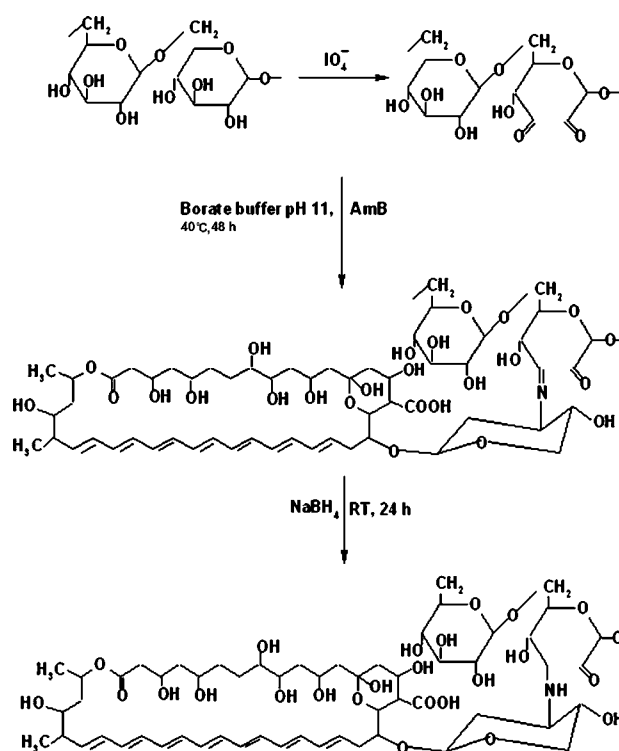
In Vitro Anti-Leishmanial Activity

The anti-leishmanial screening was done in flat-bottomed 96-well plastic tissue culture trays (Nunclon Delta, Polylabo, Strasbourg, France) maintained at 27°C in an atmosphere of 95% air and 5% CO₂.

Promastigotes were grown in M-199 medium supplemented with 40 mM HEPES, 100 µM adenosine, 0.5 mg/l hemin, 10% heat-inactivated fetal bovine serum (FBS) and 50 µg/ml gentamycin at 26°C in a dark environment. The experiments were performed with parasites in their logarithmic phase suspended to yield 10⁶ cells/mL after hemocytometer counting. Each well was filled with 100 µL of parasite suspension, and the plates were incubated at 27°C for 1 h before drug addition. The AmB conjugates to be tested were dissolved in complete M-199 medium and then added to each well to obtain the final concentration of 100 µM and further concentrations were twice diluted. Pure AmB was dissolved in DMSO and used as reference compound. Each concentration was screened in triplicate. The viability of promastigotes was checked using the tetrazolium dye (MTT) colorimetric method. After incubation of the cells with MTT reagent, a detergent solution was added to lyse the cells and to solubilize the colored crystals. The samples were read using an ELISA plate reader (Multiskan MS, Labsystem, Helsinki, Finland) at a wavelength of 570 nm. The amount of color produced was directly proportional to the number of viable cells. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC₅₀) after a 3-day incubation period (20).

In Vitro Anti-Fungal Activity

Two yeast strains *C. albicans* and *C. neoformans* were used for susceptibility testing. The minimum inhibitory concentrations (MIC) were determined by the broth dilution method according to the recommendation of the National Committee for Clinical Laboratory Standards (21). RPMI 1640 medium was buffered to a final pH of 7.0 with 0.165 M morpholinepropane sulfonic acid and 1 M NaOH, which was filter sterilized. A solution of 5 mg/mL was prepared in DMSO for free AmB and in water for AmB conjugates immediately before use. Yeast inocula were prepared from 24 h (*C. albicans*) or 48 h (*C. neoformans*) cultures grown on



Scheme 1. Synthesis of gum arabic-AmB conjugate.

Sabouraud Dextrose Agar (SDA) plates and inoculated into RPMI 1640 broth medium to yield a final inoculum concentration of 10^5 yeast cells/mL and checked by doing a viable colony count on SDA plates. Two wells containing drug-free medium and inoculum were used as controls. The inoculated plates were incubated at 35°C for 24 h (*C. albicans*) or 48 h (*C. neoformans*). The growth in each well was then estimated visually. The MIC was defined as the lowest drug concentration that resulted in complete inhibition of visible growth. The minimum fungicidal concentration (MFC) was determined by assaying the number of colony forming units (CFU) on SDA plates. The MFC was established as the lowest concentration of drug with which subcultures were negative (12).

Estimation of Plasma Drug Concentration

AmB-gum arabic conjugates were dissolved in 5% dextrose and were administered intravenously through marginal ear vein of female New Zealand white rabbits weighing about 2 kg, at a dosage of 5 mg AmB/kg body weight. Rabbits were kept in separate cages. A group of three animals was given the imine conjugate; another group of three animals was given the amine conjugate, and a third

group of three animals which did not receive any drug served as control. Rabbits were fed with commercial rabbit feed and water ad libitum. Blood samples were withdrawn from the ear vein at intervals of 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 24 h. Blood samples were stored at -80°C . AmB was extracted from plasma using a mixture of DMSO and methanol (2:1) for a period of 1 h, concentrated and analyzed by HPLC with UV detection at 405 nm (22). The column used was same as that used in estimation of AmB in the internal organs.

Statistical Analysis

Statistical analysis of data was performed by one way analysis of variance (ANOVA) assuming a confidence level of 95% ($p < 0.05$) for statistical significance.

RESULTS AND DISCUSSION

Gum arabic is a branched polysaccharide having a molecular weight (MW) of about 250,000 Da. The AmB-gum arabic conjugates were obtained in 75–80% yield. The synthesis is shown in Scheme 1. These conjugates were found

Table I. Weight Average Molar Mass M_w , of Gum Arabic and AmB-Gum Arabic Conjugates

Sample	M_w (g/mol)	Polydispersity M_w/M_n
Gum arabic	309,000	1.39
Gum arabic dialdehyde (20% oxidation)	481,836	1.4
AmB-gum arabic imine conjugate	326,227	1.29
AmB-gum arabic amine conjugate	371,535	1.29

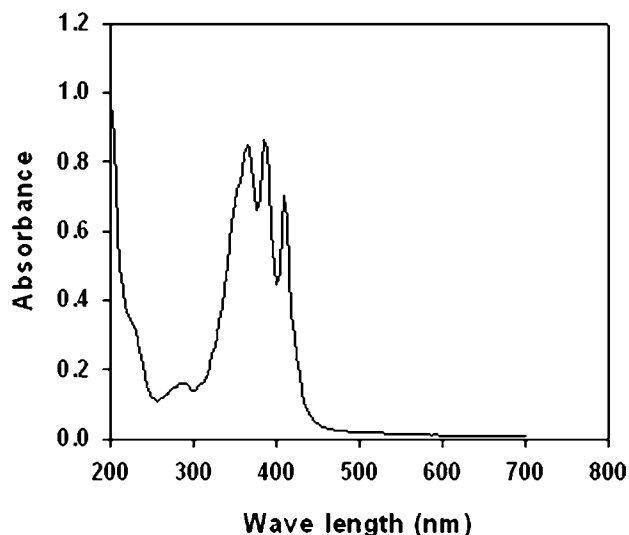


Fig. 1. UV-visible spectrum of AmB-gum arabic imine conjugate containing 20wt.% AmB.

to be water-soluble unlike AmB which has negligible solubility in aqueous solutions. Solubility was also checked in phosphate buffer, plasma and serum. The imine conjugate was found to be more soluble than the amine conjugate. The imine conjugate showed 11%, 7.24% and 3.36% solubility in phosphate buffer, plasma and serum respectively while the amine conjugate showed a solubility of 8.2%, 1.28% and 1.24%. A possible explanation for the decreased solubility of the amine conjugate is the formation of hydrogen bonds with newly formed secondary amino group in the conjugate with the hydroxyl groups in the polysaccharide.

The MW of the conjugates was determined by GPC. The MW of gum arabic, oxidized gum arabic and the conjugates are shown in Table I. It was observed that the oxidized polysaccharide had a higher MW than the parent gum. Domb et al. (13) has reported a similar phenomenon when arabinogalactan was oxidized with periodate and this increase has been

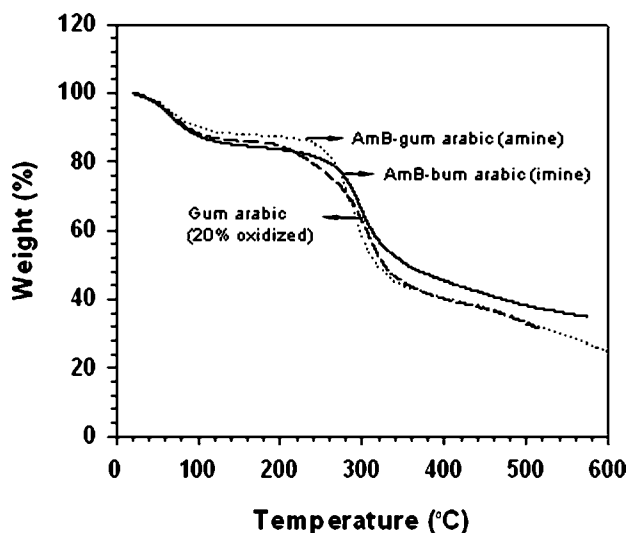


Fig. 2. Thermogram of AmB-gum arabic conjugates. Gum arabic with degree of oxidation 20% (GA-20), AmB-gum arabic imine conjugate (GA-AmB) and AmB-gum arabic amine conjugate (GA-AmBR) containing 20wt.% AmB.

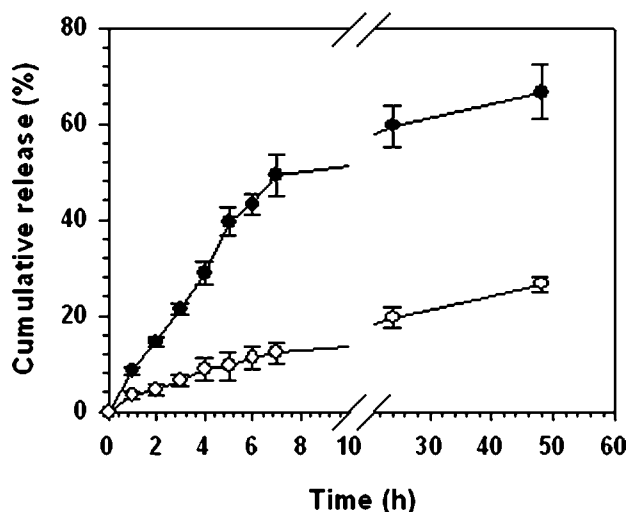


Fig. 3. *In vitro* release of AmB from AmB-gum arabic conjugates into PBS. Imine (filled circle), and amine (empty circle) conjugates containing 20wt.% AmB. Error bars represent standard deviation of three experiments.

attributed to the formation of interchain hemiacetals between hydroxyl groups and newly formed aldehyde functionalities in the oxidized polysaccharide. The MW of the imine conjugate was comparable to that of gum arabic, but less than that of 20% oxidized gum arabic. In the process of conjugate formation, the aldehyde groups are utilized in the formation of imine linkages with AmB thus breaking interchain hemiacetal linkages and reducing the MW. The MW of the amine conjugate obtained was slightly higher than that of the imine conjugate. Again, a possible explanation for this observation is the possibility of formation of hydrogen bonds with newly formed secondary amino group in the conjugated molecule.

It is reported that AmB is highly toxic in its aggregated state than in its monomer form (23). In solution, AmB exists in three different forms—monomers, oligomers and aggregates. Soluble form of AmB exists in monomeric form. Monomeric

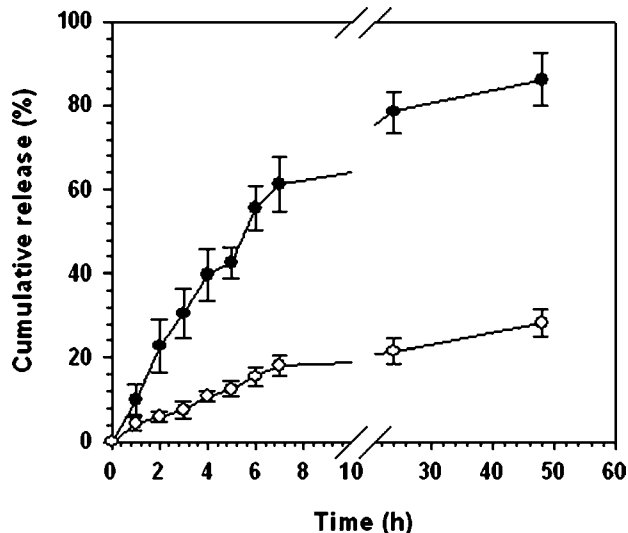


Fig. 4. *In vitro* release of AmB from AmB-gum arabic conjugates into 0.9 mM BSA solution. Imine (filled circle), and amine (empty circle) conjugates containing 20wt.% AmB. Error bars represent standard deviation of three experiments.

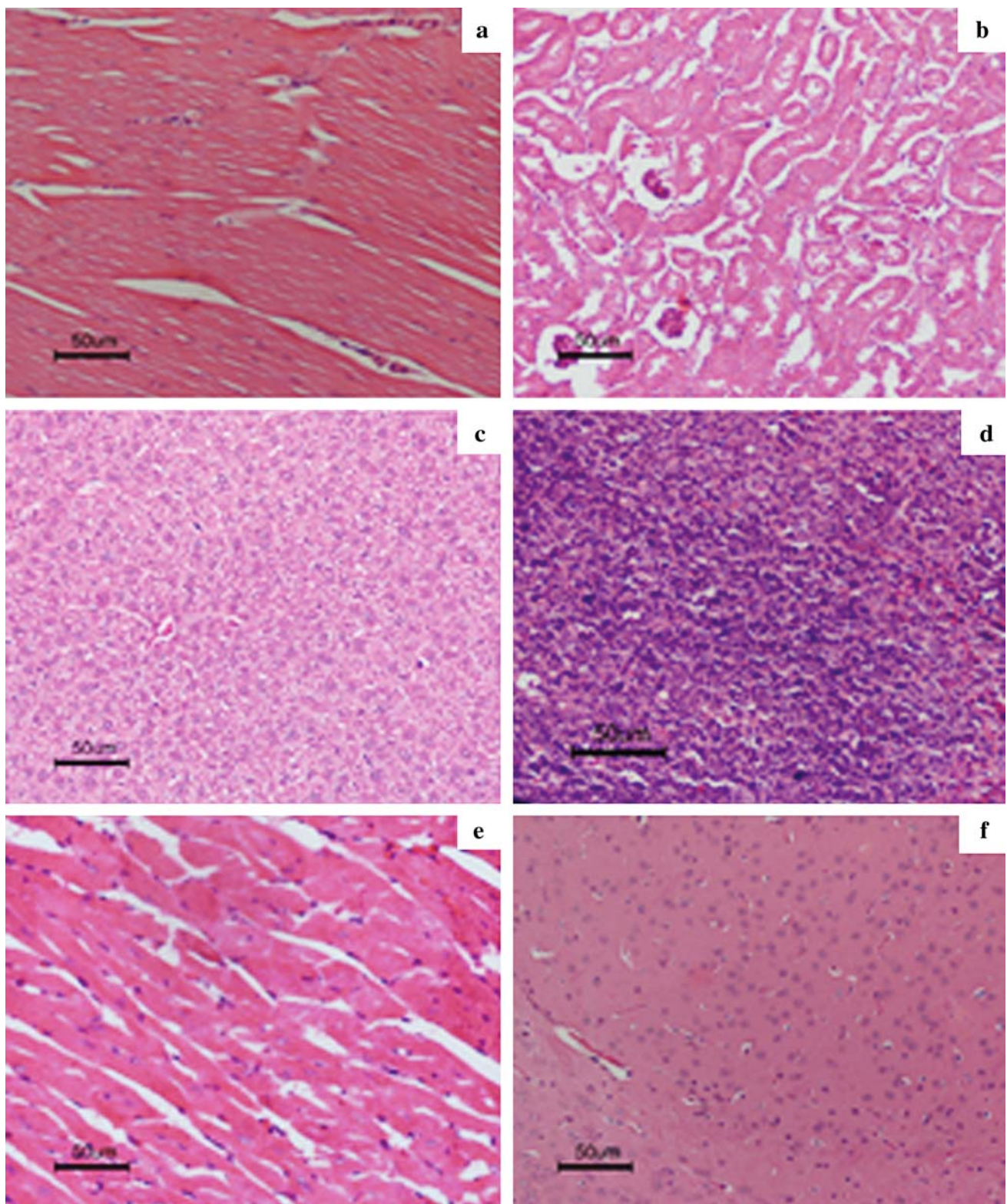


Fig. 5. Histomorphology of internal organs of mice that received AmB-gum arabic imine conjugate. **a**-Muscle, **b**-Kidney, **c**-Liver, **d**-Spleen, **e**-Heart, **f**-Brain.

AmB associates with ergosterol in fungal cell membranes whereas aggregated AmB can also form pores in sterol containing membranes, leading to toxicity towards host cell (24). The amount of aggregation was determined using UV-

Visible spectrophotometry. Fig. 1 shows the UV-visible spectrum of AmB-gum arabic imine conjugate in PBS (0.1 M, pH 7.4) at a concentration of 0.05 g/mL soon after preparation. The UV-visible spectra of polyenes are very sensitive to conforma-

Table II. Anti-Leishmanial Activity of AmB-Gum Arabic Conjugates against Promastigotes of *L. donovani*

	<i>L. donovani</i> LV9 IC ₅₀ (μM±SD)	<i>L. donovani</i> DD8 IC ₅₀ (μM±SD)
AmB formulations ^a		
20(20)	0.37±0.04	1.44±0.18
20(20)R	8.54±0.11	2.56±0.31
Reference compounds		
AmB	0.15±0.02	0.09±0.01
GA(20)	> 0.5 mg/mL	> 0.5 mg/mL

^a 20(20) is 20% oxidized gum arabic having 20% AmB as the imine conjugate and 20(20)R is 20% oxidized gum arabic having 20% AmB in the reduced form as the amine conjugate. GA(20) is 20% oxidized gum arabic.

tional changes induced by different molecular interactions, including aggregation. The ratio of absorbance at 348 nm to 409 nm is reported to give the extent of aggregation in AmB (25). Barwicz et al. (23) report the ratio to be 2 for aggregated species. Most of the AmB formulations have greater A_{348}/A_{409} values. For instance, Fungizone[®] has a value of 2.9 while AmBisome[®] has a value of 4.8 (26). For AmB-gum arabic conjugate, the value obtained was 0.96 showing that AmB was not aggregated in the conjugate.

The thermal stability of the conjugates was examined using thermogravimetry. Gum arabic aldehyde started to degrade at 156.54°C, and degradation took place in four stages, and after 596.16°C, only 10.47% of the material was left. Degradation of AmB-gum arabic imine conjugate took place in three stages; it started to degrade at 170.90°C and after 590.71°C, 39.24% of the material was left. Amine conjugate of AmB-gum arabic degraded in three stages. Degradation started at 133.80°C, and after 594.44°C, 14.53% of the material was left. The conjugates show better thermal stability and degrade at a slower rate compared to gum arabic aldehyde (Fig. 2). The imine and amine linkages introduced are believed to exert a stabilizing effect than the weak hemiacetal linkages between the aldehyde and hydroxyl groups present in the oxidized polysaccharide thereby improving the thermal stability of the conjugates.

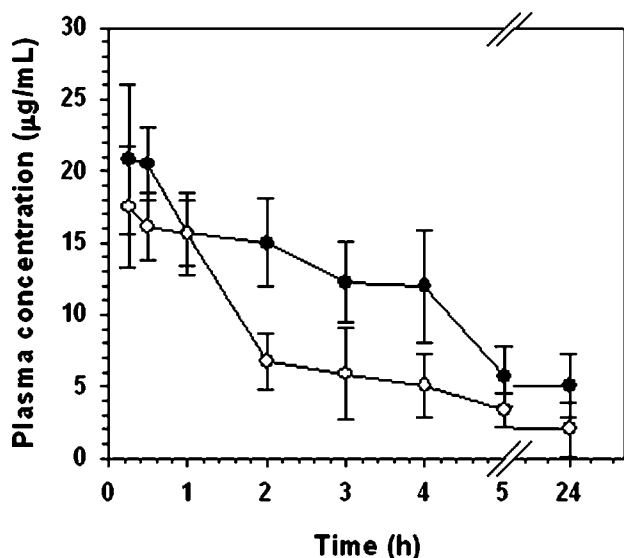


Fig. 6. Plasma distribution of AmB from imine (filled circle) and amine (empty circle) conjugates of gum arabic containing 20wt.% AmB in rabbits after intravenous administration. Error bars represent standard deviation of three estimations.

Determination of the hemolytic potential of the conjugates showed that both amine and imine conjugates were not hemolytic at concentrations equivalent to 700 μg AmB/mL whereas pure AmB prepared from a stock solution in DMSO was hemolytic, and caused 50% hemolysis at a concentration of 3.5 μg/mL as reported earlier (11). Thus, the conjugated polymer possibly exerts a stabilizing and protective effect on the drug.

In vitro release of AmB from both imine and amine conjugates was examined in PBS as well as in an *in vivo* 'model' solution of 0.9 mM BSA. Figs. 3 and 4 show the release profiles from both conjugates into PBS and BSA solution respectively. Release from imine conjugate was faster into both media whereas release from amine conjugate was considerably slowed down. The hydrolytic susceptibility of the imine bond linking AmB to the polysaccharide is evident in the release profile. The amine bond, as expected is more stable. Release from both imine and amine conjugates was not significantly different in both media ($p < 0.5$). It is thus evident that the AmB coupled onto the polysaccharide would be released by hydrolysis.

The toxicity of the conjugates was evaluated in mice. Mice were able to withstand a dosage of 20 mg AmB/kg of both conjugates. Immediate death of the animal was observed when a dosage of 100 mg AmB/kg was injected. When the animal was given a dosage of 50 mg AmB/kg, the animal survived for 1 day. Similarly, when the animal was given a dosage of 35 mg AmB/kg it survived for 2 days. Mice ($n=10$) which received a dosage of 20 mg AmB/kg survived uneventfully for 7 days when they were sacrificed to examine the internal organs for the presence of AmB and for histological evaluation.

AmB is reported to be highly neurotoxic and nephrotoxic (27). It is also reported to be toxic to liver and heart (26,28). Therefore, the internal organs; brain, kidney, liver, heart, spleen and muscles of mice that received maximum tolerable dosage was observed for morphological changes. Fig. 5 shows representative photographs of the histomorphology of various organs of mice that received the AmB-gum arabic imine conjugate. Control samples taken from mice that did not receive any drug were also observed under same conditions. Histomorphology of the test samples showed the same features as that of control samples. Lesions were not detected in kidney, brain, heart or liver suggesting AmB-gum arabic conjugate did not exhibit any nephrotoxic, neurotoxic, cardiotoxic or hepatotoxic effects. Incidence of AmB nephrotoxicity is reportedly very high and acute renal failure is common (6). It is reported that kidneys of mice treated with AmB-deoxycholate exhibited tubular necrosis and liver showed single-cell necrosis and

Table III. Pharmacokinetic Parameters of AmB-Gum Arabic Conjugates Administered Intravenously to Rabbits^a

AmB 5 mg/kg	AUC (min.µg/mL)	CL (mL/min)	k_{el} (min ⁻¹) x10 ³	$t_{1/2}$ (min)	V_{ss} (mL)	MRT (min)
Imine	9,936	0.503	2.534	273.4	198.50	394.63
Amine	5,417	0.923	6.165	112.4	149.72	162.21

^a AUC, area under the concentration-time curve, CL, clearance, k_{el} , elimination rate constant. $t_{1/2}$, elimination half life, V_{ss} , volume distribution, MRT, mean residence time.

Table IV. MICs and MFCs of AmB-Gum Arabic Conjugates

Sample	<i>C. albicans</i>		<i>C. neoformans</i>	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Imine conjugate	0.5–0.9	0.9	0.5–0.9	0.9
Amine conjugate	0.5–0.9	0.9	0.5–0.9	0.9
AmB	0.4	0.4	0.2	0.2

neutrophil aggregation (12). No such complications were present in the internal organs of mice treated with AmB-gum arabic conjugate.

In order to examine whether AmB is still retained in the organs after 7 days of the maximum tolerated dose of a single infusion of the imine conjugate equivalent to 20 mg AmB/kg, HPLC analysis of extracts from the organs was carried out. Interestingly, while organs such as kidney, liver and the lung were found to contain less than 1 µg of AmB per gram of tissue, the spleen still retained 8.4±0.53 µg/g showing that the highest concentration of AmB after administration of the conjugate occurred in spleen. Repeated infusions of 5 mg/kg of AmB-arabinogalactan conjugate for five consecutive days by Domb *et al.* (15) have shown that the concentrations were much higher in the lungs than in the spleen at 7 days. Possibly, the MW of the polymer exerts a significant influence in determining the organ distribution of the conjugate since gum arabic has an average MW ten times that of arabinogalactan. This however, needs more extensive investigation. As AmB is getting more accumulated in spleen where the leishmanial parasites reside, possibly, this conjugate would be effective for anti-leishmanial chemotherapy.

The anti-leishmanial activity of both imine and amine conjugates was examined against the promastigotes of *L. donovani* LV9 and DD8. The imine conjugate showed lower

IC₅₀ values for both strains whereas for the amine conjugate the values were much higher (Table II). The imine linkages in Schiff's base are less stable and more prone to hydrolysis than the more stable amine linkages that are less prone to hydrolysis. The difference in reactivity could therefore be attributed to the stability of the polymer-drug bond in these conjugates. It is however interesting to note that the amine conjugates are not without reactivity, they are active at much higher concentrations as compared to their imine counterparts. Domb *et al.* (14) found that both imine and amine conjugates of arabinogalactan were active against promastigotes of *L. major* and *L. infantum* although the amine conjugate required approximately twice the concentration of the imine conjugate.

The *in vivo* bioavailability of AmB from the conjugates was examined by intravenous injection of the conjugates and examining the concentration of AmB in the blood at various time intervals. The pharmacokinetic parameters were calculated from the plasma concentration-time curve (Fig. 6). AmB-gum arabic imine conjugate when administered intravenously at a dose of 5 mg AmB/kg body weight in rabbits gave an initial plasma concentration of 20 µg/mL. Steady concentration of AmB in plasma was attained after 1 h, and the plasma concentration was stabilized at 15 µg/mL for 4 h. It decreased to 5 µg/mL in 5 h and this concentration was

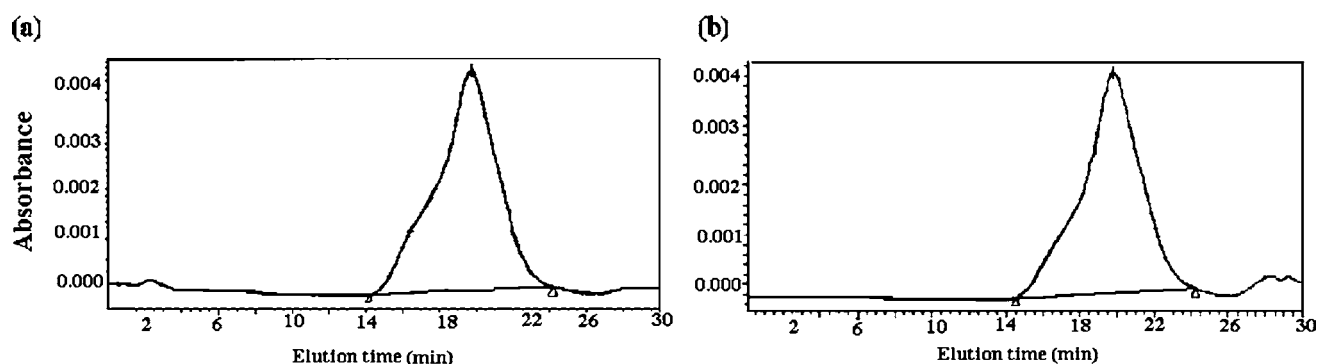


Fig. 7. GPC of the gum arabic-AmB imine conjugates containing 20wt.% AmB. After synthesis (a) and after 8 months of storage (b).

maintained for 24 h. With the amine conjugate, the concentration of AmB was slightly less, initial plasma concentration attained was 17 $\mu\text{g/mL}$, which decreased to 6 $\mu\text{g/mL}$ in 2 h. There was further steady decrease in plasma concentration and it reached 3 $\mu\text{g/mL}$ in 24 h. Statistical analysis showed that the plasma concentration attained from both imine and amine conjugates was significantly different ($p < 0.5$). AmB-arabinogalactan conjugate when administered to mice at a dosage of 5 mg/kg was reported to give an initial plasma concentration of 7 $\mu\text{g/mL}$ which climbed down to 2.5 $\mu\text{g/mL}$ in 24 h (15). These values are strictly not comparable since the animal species employed in both investigations are different. The pharmacokinetic parameters are shown in Table III. The results showed that availability of drug is more from the imine conjugate than from the amine conjugate. Elimination rate constant is higher for the amine conjugate, compared to imine conjugate and its elimination half life is also shorter. The amine conjugate is more resistant to hydrolysis compared to the imine conjugate which explains its lower availability. Thus, although the MW of the polysaccharide employed is about ten times that of arabinogalactan, it was interesting to note that AmB is bioavailable which points out the cleavage of the polymer-drug bond during systemic circulation. Oxidized polysaccharides are also susceptible to rapid degradation compared to their unoxidized counter parts (29,30) and this also possibly contributes to the bioavailability of the drug from the high molecular weight polymer.

AmB-gum arabic conjugates were also evaluated against *C. albicans* and *C. neoformans* for anti-fungal activity (Table IV). MIC was in the range of 0.5–0.9 $\mu\text{g/mL}$ for both imino and amino conjugates of AmB and MFC was 0.9 $\mu\text{g/mL}$. For pure AmB (in DMSO), MIC and MFC values were 0.4 $\mu\text{g/mL}$. When compared to pure AmB, both the conjugates were less active. AmB is conjugated to gum arabic by the amino function. The amino group of AmB is known to participate in the formation of hydrogen bond between the polyene and the sterol (5). Thus, functionalization of amino group may result in decreased polyene concentration to induce permeability, but the hydrolysis of conjugates could release AmB for pharmacological interaction with membrane. However, activity of AmB-gum arabic conjugate is comparable with AmB-arabinogalactan conjugate, which has an MFC value of 0.5–1.0 $\mu\text{g/mL}$ for *C. albicans* and 0.25–1.00 $\mu\text{g/mL}$ for *C. neoformans* (12).

The storage stability of the AmB-gum arabic imino conjugate was examined. Conjugate prepared from 20% oxidized gum arabic containing 20 wt.% AmB was stored in the dark at 4°C for 8 months. The chemical stability of the conjugates was determined by GPC, with UV absorption at 405 nm using samples freshly prepared at a concentration of 0.5 mg/mL in distilled water. Intensity of the peaks remained same after storage, showing that they do not degrade on storage (Fig. 7).

CONCLUSIONS

AmB conjugated to a high molecular weight polysaccharide such as gum arabic was still found to be non-hemolytic, non-toxic and exhibited anti-fungal and anti-leishmanial activity confirming the release of AmB from the conjugates

as demonstrated in the *in vitro* release studies. Interestingly, in spite of the high molecular weight of the polysaccharide, AmB was still bioavailable when given by the intravenous route. Estimation of the residual drug in the internal organs of mice 7 days after a single injection showed that the spleen still retained maximum drug concentration demonstrating potential for anti-leishmanial therapy. The conjugates were found to be stable on storage as lyophilized powder for 8 months. Gum arabic, a highly branched polysaccharide widely used in food and pharmaceutical industry is also less expensive and therefore would be good candidate polymer for *polymer therapeutics*. Since the oral efficacy of AmB remains a challenge for leishmaniasis mass treatment, further studies will be focused on the antileishmanial potential of these conjugates when administered by oral route to Balb/c mice infected with *L. donovani*.

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REFERENCES

1. A. H. Groll and T. J. Walsh. Uncommon opportunistic fungi: new nosocomial threats. *Clin. Microbiol. Infect.* **7** (Suppl 2):8–24 (2001).
2. T. J. Walsh, J. Hiemenz, and E. Anaissie. Recent progress and current problems in treatment of invasive fungal infections in neutropenic patients. *Infect. Dis. Clin. North Am.* **10**:365–400 (1996).
3. H. A. Gallis, R. H. Drew, and W. W. Pickard. Amphotericin B: 30 years of clinical experience. *Rev. Infect. Dis.* **12**:308–329 (1990).
4. J. D. Berman. Human leishmaniasis: clinical, diagnostic and chemotherapeutic developments in past 10 years. *Clin. Infect. Dis.* **24**:684–703 (1997).
5. J. Brajtburg, W. G. Powderly, G. S. Kobayashi, and G. Medoff. Mini review. Amphotericin B: current understanding of mechanism of action. *Antimicrob. Agent Chemother.* **34**:183–188 (1990).
6. G. Deray. Amphotericin B nephrotoxicity. *J. Antimicrob. Chemother.* **49**:37–41 (2002).
7. J. Brajtburg and J. Bolard. Carrier effects on biological activities of Amphotericin B. *Clin. Microbiol. Rev.* **9**:512–531 (1996).
8. B. Dupont. Overview of the lipid formulations of amphotericin B. *J. Antimicrob. Chemother.* **49** (suppl S1):31–36 (2002).
9. V. Yardley and S. L. Croft. Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. *Antimicrob. Agents Chemother.* **41**:752–756 (1997).
10. S. L. Croft and V. Yardley. Chemotherapy of Leishmaniasis. *Curr. Pharm. Des.* **8**:319–342 (2002).
11. M. Larabi, V. Yardley, P. M. Loiseau, M. Appel, P. Legrand, A. Gulik, C. Bories, S. L. Croft, and G. Barrat. Toxicity and Antileishmanial activity of a new stable lipid suspension of amphotericin B. *Antimicrob. Agents Chemother.* **47**:3774–3779 (2003).
12. R. Falk, A. J. Domb, and I. Polacheck. A novel injectable water-soluble amphotericin B-arabinogalactan conjugate. *Antimicrob. Agents Chemother.* **43**:1975–1981 (1999).
13. E. Kleinman, T. Azzam, R. Falk, I. Polacheck, J. Golenser, and A. J. Domb. Synthesis and characterization of novel water

- soluble amphotericin B-arabinogalactan conjugate. *Biomaterials* **23**:1327–1335 (2002).
14. J. Golenser, S. Frankenburg, T. Ehrenfreund, and A. J. Domb. Efficacious treatment of experimental leishmaniasis with amphotericin B-arabinogalactan water-soluble derivatives. *Antimicrob. Agents Chemother.* **43**:2209–2214 (1999).
 15. R. Falk, J. Grunwald, A. Hoffman, A. J. Domb, and I. Polacheck. Distribution of amphotericin B-arabinogalactan conjugate in mouse tissue and its therapeutic efficacy against murine aspergillosis. *Antimicrob. Agents Chemother.* **48**:3606–3609 (2004).
 16. N. Matsumori, N. Eiraku, S. Matsuoka, T. Oishi, M. Murata, T. Aoki, and T. Ide. An amphotericin B-ergosterol covalent conjugate with powerful membrane permeabilizing activity. *Chem. Biol.* **11**:673–679 (2004).
 17. E. Charvalos, M. N. Tzatzarakis, F. V. Bambeke, P. M. Tulkens, A. M. Tsatsakis, G. N. Tzanakakis, and M.-P. Mingeot-Leclercq. Water-soluble amphotericin-B-polyvinylpyrrolidone complexes with maintained antifungal activity against *Candida* spp and *Aspergillus* spp and reduced haemolytic and cytotoxic effects. *J. Antimicrob. Chemother.* **57**:236–244 (2006).
 18. D. Verbeken, S. Dierckx, and K. Dewettinck. Mini-review: exudate gums: occurrence, production, and applications. *Appl. Microbiol. Biotechnol.* **63**:10–21 (2003).
 19. K. K. Nishi and A. Jayakrishnan. Preparation and in vitro evaluation of primaquine-conjugated gum arabic microspheres. *Biomacromolecules* **5**:1489–1495 (2004).
 20. S. Raynaud-Le Grandic, C. Fourneau, A. Laurens, C. Bories, R. Hocquemiller, and P. M. Loiseau. In vitro antileishmanial activity of acetogenins from annonaceae. *Biomed. Pharmacother.* **58**:388–392 (2004).
 21. National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M-27A*, National Committee for Clinical Laboratory Standards, Wayne, PA, 1997.
 22. T. J. Walsh, A. J. Jackson, J. W. Lee, M. Amantea, T. Sein, J. Bacher, and L. Zech. Dose-dependent pharmacokinetics of amphotericin B lipid complex in rabbits. *Antimicrob. Agents Chemother.* **44**:2068–2076 (2000).
 23. J. Barwicz, S. Christian, and I. Gruda. Effects of aggregation of amphotericin B on its toxicity to mice. *Antimicrob. Agents Chemother.* **36**:2310–2315 (1992).
 24. M. Larabi, A. Gulik, J. P. Dedieu, P. Legrand, G. Barrat, and M. Cheron. New lipid formulations of Amphotericin B: spectral and microscopic analysis. *Biochem. Biophys. Acta.* **1664**:172–181 (2004).
 25. P. Legrand, E. A. Romero, B. E. Cohen, and J. Bolard. Effects of aggregation and solvent on the toxicity of amphotericin B to human erythrocytes. *Antimicrob. Agents Chemother.* **36**:2518–2522 (1992).
 26. A. B. Mullen, K. C. Carter, and A. J. Baillie. Comparison of the efficacies of various formulations of amphotericin B against murine visceral leishmaniasis. *Antimicrob. Agents Chemother.* **41**:2089–2092 (1997).
 27. M. S. Maddux and A. L. Barriere. A review of complications of amphotericin B therapy: Recommendations for prevention and management. *Drug. Intel. Clin. Pharm.* **14**:177–181 (1980).
 28. P. J. Danaher, M. K. Cao, G. M. Anstead, M. J. Dolan, and C. C. DeWitt. Reversible dilated cardiomyopathy related to amphotericin B therapy. *J. Antimicrob. Chemother.* **53**:115–117 (2004).
 29. K. Y. Lee, K. H. Bouhadir, and D. J. Mooney. Degradation behaviour of covalently cross-linked poly(aldehyde guluronate) hydrogels. *Macromolecules* **33**:97–101 (2000).
 30. T. Boontheeikul, H.-J. Kong, and D. J. Mooney. Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials* **26**:2455–2465 (2005).