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Nanosomal Amphotericin B is an efficacious alternative to Ambisome® for fungal therapy

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ABSTRACT

Amphotericin B was formulated in lipids (Nanosomal Amphotericin B) without using any detergent or toxic organic solvents during the preparation. Electron microscopy and particle size determination of Nanosomal Amphotericin B showed a homogeneous population of nanosized particles below 100 nm. Hemolysis assay indicated that Nanosomal Amphotericin B causes significantly less lysis of red blood cells than Amphotericin B deoxycholate and was comparable to Ambisome®. A maximum daily dose of Nanosomal Amphotericin B at 5 mg/kg in rabbits and 10 mg/kg in mice for 28 days showed no symptoms of toxicity, mortality or significant body weight reduction. Hematological and gross pathological analysis of tissues revealed no abnormalities attributable to the drug treatment. Nanosomal Amphotericin B and Ambisome® were injected (iv) at 2 mg/kg consecutively for 5 days into mice infected with Aspergillus fumigatus. The treatment resulted in 90% survival with Nanosomal Amphotericin B and only 30% survival with Ambisome® after 10 days of fungal infection. However, all of the 10 control mice which were not treated with Amphotericin B died within 5 days of fungal infection. Nanosomal Amphotericin B is safe, cost effective and provides an alternative option for treatment of fungal disease.

1. Introduction

Several non-polyene drugs, such as azoles (voriconazole and posaconazole) and echinocandins (caspofungin, micafungin, and anidulafungin), are approved for the treatment of antifungal diseases, but polyenes are still used extensively because of its broad antifungal spectrum and lower incidence of developing resistance (Lanternier and Lortholary, 2008). Among polyenes, Amphotericin B has been extensively used for the treatment of severe systemic fungal diseases for the past several decades (Andrews et al., 1977; Ostrosky-Zeichner et al., 2003). In addition, studies have shown that it is also effective in the treatment of visceral leishmaniasis (Ahmad et al., 1991b; Crof et al., 1991; Brajtburg and Bolard, 1996). Since Amphotericin B is insoluble, it was originally solubilized and formulated in deoxycholate (Fungizone), a conventional formulation of Amphotericin B. Amphotericin B deoxycholate has been a gold standard for treatment of patients with invasive fungal infections (Brajtburg and Bolard, 1996). However, this colloidal form administered to patients via intravenous route results in severe side effects including fever, nausea, vomiting, lysis of red blood cells (RBCs) and nephrotoxicity and thus its maximal utilization in clinical practice is restricted. To reduce the toxic effects of Amphotericin B, several lipid-based formulations were developed and compared with the Fungizone (Gregoriadis, 1987; Szoka et al., 1987; Ahmad et al., 1990a,b; Gates and Pinney, 1993). In general, lipid-based formulations were shown to be less toxic than Fungizone. This may be due to altered pharmacological distribution and minimal interaction with RBCs (Ahmad et al., 1990b). An improved delivery of Liposomal Amphotericin B to disease site was also shown in Aspergillosis mouse model (Ahmad et al., 1989, 1990b, 1991a; Moonis et al., 1994). Presently, three lipid based Amphotericin B formulations are available in the market namely (1) Abelcet®, a ribbon like particles composed of dimyristoyl phosphotidylcholine (DMPC) and dimyristoyl phosphotidylglycerol (DMPG); (2) Amphotec®/Amphocil®, a disk-like particles composed of only cholesteryl sulfate; and (3) Ambisome®, a unilamellar liposome composed of hydrogenated soy phosphatidylcholine (HSPC), cholesterol and distearoyl phosphatidylcholine (DSPG) (Gregoriadis, 1987; Gates and Pinney, 1993; Swenson et al., 1998). In all lipid based or liposomal preparation of Amphotericin B, the

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lipids and the drug are solubilized in organic solvents. The organic solvents are finally removed using standard solvent removal methods in lipid or liposome based preparations of Amphotericin B. The use of organic solvent and its removal process is quite cumbersome and expensive. All three marketed lipid-based formulations (Ambisome®, Amphotec®, and Abelcet®) permit higher doses of drug administration compared to Fungizone.

This manuscript reports for the first time the development of a well-characterized new lipid-based formulation of Amphotericin B (Nanosomal Amphotericin B) without using any organic solvent or detergent at any step of the entire manufacturing process. Nanosomal Amphotericin B showed significantly reduced toxicity both in vitro and in vivo. In addition, Nanosomal Amphotericin B demonstrated similar hemolysis and enhanced therapeutic efficacy compared to marketed Ambisome® product.

2. Materials and methods

2.1. Materials

Amphotericin B was obtained from Alpharma (Denmark). Amphotericin B deoxycholate (Amphotericin B for Injection, 50 mg) was obtained from Sarabhai Chemicals, India (Manufactured for E.R. Squibb & Sons Ltd., UK). Ambisome® was procured from Astellas Pharma US Inc. (Deerfield, IL). Soy phosphatidylcholine (SPC) was purchased from Lipoid LLC (Newark, NJ) and sodium cholesteryl sulfate was obtained from Genzyme Pharmaceuticals (Cambridge, MA). Aspergillus fumigatus (36607) was obtained from American Type Culture Collection (Manassas, VA). A 5% dextrose solution was procured from Hospira (Lake Forest, IL).

2.2. Preparation of Nanosomal Amphotericin B

SPC (19.74 g), sodium cholesteryl sulfate (260 mg), and alpha-tocopherol (44 mg) were mixed with disodium succinate buffer (8.33 mM, pH 5.2, 700 mL) and homogenized using IKA homogenizer until the suspension was free from lumps and then passed through the Emulsiflex C3 Homogenizer (Avestin, Inc. Ottawa, Canada) 5 times at 25,000 psi. In a separate solution, the pH of disodium succinate buffer (8.33 mM, 200 mL) was adjusted to 11.7 with 1 N NaOH and Amphotericin B (2 g) was added and stirred until a clear solution was obtained. Subsequently, the pH of the Amphotericin B solution was carefully adjusted back to pH 10.0 using 1 N HCl. Amphotericin B solution was then added to the lipid suspension while stirring to provide an Amphotericin B lipid suspension yielding a pH of ~5.8. The resulting Amphotericin B lipid suspension was passed through the Emulsiflex C3 Homogenizer twice at 25,000 psi. The Amphotericin B lipid suspension was slowly added while stirring to a solution of sucrose (75 g) in disodium succinate buffer (8.33 mM) (100 mL). The resulting suspension was filtered through 0.22 μm sterile PVDF Millipak100 filter. The final Nanosomal Amphotericin B product was dispensed in vials and lyophilized to yield a concentration of Amphotericin B (50 mg per vial).

2.3. Physicochemical characterization

2.3.1. Freeze-fracture electron microscopy

The lyophilized product was reconstituted in water for injection (WFI) and the particle size was checked using the Nicomp Model 380/ZLS&S Potential/Sub-Micron Particle Sizer (Particle Sizing Systems, New Port Richly, Florida). The measurements were carried out at 23 °C at a scattering angle of 90°. The lyophilized products were analyzed and found to be endotoxin free.

Nanosomal Amphotericin B morphology was characterized by freeze-fracture electron microscopy. The samples were quenched using the liquid nitrogen-cooled and propane sandwich technique. The cryo-fixed samples were stored in liquid nitrogen for less than 2 h before processing. The fracturing process was carried out in the JEOL JED-9000 freeze-etching equipment and the exposed fracture planes were shadowed with platinum for 30 s at an angle of 25–35° and with carbon for 35 s (2 kV/60–70 mA, 1×10^{-5} Torr). The replica produced was cleaned with concentrated, fuming HNO_3 for 24 h followed by repeating agitation with fresh chloroform/methanol (1:1 by vol.) at least 5 times. The clean replica was examined at JEOL 100 CX TEM/Micro analytical laboratories, Inc. and at JEOL 1230 TEM/Stanford University with a digital camera.

2.3.2. HPLC method

Amphotericin B concentration in Nanosomal Amphotericin B formulation was determined by a prepacked YMC Pack Pro C18 column (YMC, Europe, GmbH, 3 μm particle size, 150 mm × 4.6 mm i.d.) attached with Agilent 1100/1200 Series HPLC systems (Agilent Technology, Palo Alto, CA) and a UV detector. The column temperature was set at 20 °C and 20 μL of sample volume were injected into the HPLC for analysis at 383 nm. The HPLC was run using mobile phases consisting of methanol: acetonitrile: 21.8 mM citric acid–ammonia (pH 4.7) (10:30:60, mobile phase A) and methanol: acetonitrile: 21.8 mM citric acid–ammonia (pH 3.9) (12:68:20, mobile phase B) at a flow rate of 0.8 mL/min with 3 min at 0% B followed by a 20 min linear gradient from 0 to 30% B. Amphotericin B was eluted at a retention time of about 18.0 min.

2.4. Preparation of dosing solutions

The Nanosomal Amphotericin B and Ambisome® vials each containing 50 mg Amphotericin B were first diluted to 2 and 4 mg/mL concentration with the sterile water, respectively. Aliquots of each of these two solutions were further diluted with 5% dextrose solution to prepare the dosing solutions of Amphotericin B 0.2 mg/mL concentration for the intravenous administration to the fungal infected mice.

2.5. Animal toxicology

New Zealand white rabbits were obtained from Serum Institute of India Ltd., Pune, India. The rabbits were housed individually in stainless steel cages with stainless steel mesh bottom. The temperature was kept at 22 ± 3 °C with 30–70% relative humidity and 12 h each of dark and light cycle were maintained. Animals were acclimated to the laboratory conditions for 7 days prior to dosing. Each animal was identified using picric acid. The females were nulliparous and non-pregnant. The animal housing and study was performed in accordance with the Schedule Y of Drugs and Cosmetic Act, India (IInd Amendment) Rules, 2005 and regulations of the Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA, Indian Institute of Toxicology, Pune 411013, India, Registration No. 15/19999/CPCSEA).

Male and female rabbits (n = 3/sex/group) were administered with 0, 1, 2.5 and 5 mg/kg of Nanosomal Amphotericin B intravenously daily for 28 days. The control animals were administered with 5% dextrose. The weekly food consumption was recorded and calculated for each animal in the control and treatment groups. At the end of the study (day 29), all the animals were sacrificed. Necropsy of all animals was carried out and the weights of the following organs were recorded: liver, kidneys, adrenals, spleen, brain, heart, lungs, thymus, testes/ovaries and epididymis/uterus. The organ weights were recorded as absolute values and their relative values (i.e. percent of the body weight) were calculated. Further hematological, biochemical analysis, gross pathological and histopathalogical analyses were conducted.

The following organ tissue samples from control and treated animals were preserved in 10% formalin for histopathological

examination: adrenals, aorta, brain, caecum, colon, duodenum, epididymis, eyes, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, skin, oesophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary glands, sciatic nerve, seminal vesicles, skeletal muscle, spleen, spinal cord, sternum with bone marrow, stomach, testes, thymus, thyroid/parathyroid, trachea, urinary bladder and uterus.

2.6. RBC hemolysis

The hemolysis activity was measured by the method described by Ahmad et al. (1995). Whole blood collected from the healthy human subject(s) was centrifuged for 5 min at 3000 rpm. The supernatant containing the plasma fraction was removed, and the RBCs were re-suspended in phosphate buffered saline (10 mM sodium phosphate, pH 7.4 containing 0.9% NaCl, PBS). After centrifugation for another 5 min at 3000 rpm, the washed RBCs were re-suspended in PBS to prepare a 4% RBCs (v/v) solution. The Amphotericin B formulations (Nanosomal Amphotericin B, Ambisome® and Amphotericin B deoxycholate) and placebo of Nanosomal Amphotericin B at various concentration or PBS were mixed with an equal volume of RBCs. Hemolysis of RBCs in water was used as a 100% lysis control. The microtiter plate containing the samples for hemolysis was constantly agitated for 1 h at 37 °C. The plate was centrifuged at 3000 rpm for 10 min and an aliquot of supernatant was taken to measure the hemoglobin content at 550 nm using the SpectraMax M2e (Molecular Devices, CA).

2.7. Animal model for Aspergillosis

Aspergillus fumigatus strain (Lot# 58448831) was harvested in the Sabouraud Dextrose pH 5.6-Slants for about 4 days. The harvested fungal spores were isolated using a sterilized n-saline/0.2% Tween-80 solution, and the fungal suspension was filtered through sterilized gauze. The filtered spores were counted using a hemocytometer after appropriate dilution. The fungal suspension was adjusted to $\sim 10 \times 10^7$ spores per mL suspension with sterilized normal saline containing 0.2% Tween-80. Three groups of 10 ICR (CD-1) mice (Harlan Laboratories, Madison, Wisconsin) each were administered a volume of 0.2 mL solution ($\sim 2 \times 10^7$ spores) intravenously into the tail vein. The Aspergillus fumigatus mice model was optimized by intravenously injecting 2×10^7 spores at three different experiments producing approximately the same mortality rate at and after 48 h. This dose caused disseminated fungal infection and the mice survived for 2 days providing sufficient time for the evaluation of therapeutic efficacy. The guidelines for the Care and Use of Laboratory Animals and Standard Operating Procedures for animal well-being were followed.

2.8. Treatment of mice infected with Aspergillus fumigatus

The mice (4–6 weeks old) were group housed in polycarbonate shoebox cages (27 cm × 21 cm × 14 cm) with a wire feeder and 250 mL water bottle (Techniplast). The animal room temperature was kept in between 20 and 25 °C with 20–30% relative humidity and 12 h each of dark and light cycle were maintained. Water and feed (18% protein rodent diet, Harlan Laboratories) were provided *ad libitum*. The mice were randomly divided into one control and two test groups; each containing 10 mice. The mice were infected with Aspergillus fumigatus as described above. The 5 days of intravenous dosing of either 5% dextrose (control), Nanosomal Amphotericin B or Ambisome® solutions was performed at ~5, 24, 48, 72 and 96 h post fungal infection to the surviving mice, as applicable. The dose rate was 2 mg Amphotericin B/kg body weight/day for the two test groups. The efficacy of Nanosomal Amphotericin B and Ambisome® was evaluated on the basis of survival of mice. Survival was checked daily for up to 10 days post fungal infection.

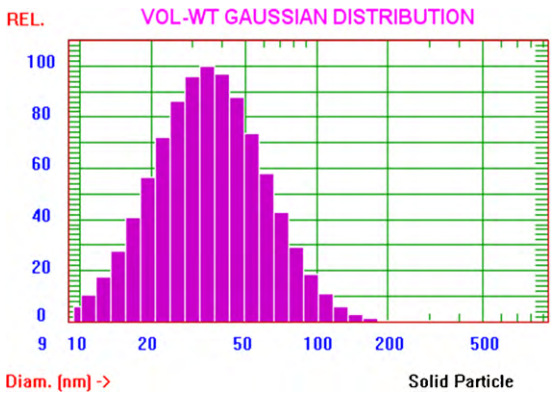

Fig. 1. Particle size distribution of Nanosomal Amphotericin B: mean particle size, 40.2 nm, distribution (<%) D25 24.6 nm, D50 35.1 nm, D75 50.5 nm, D80 55.2 nm, and D99 123.5 nm. The measurements were carried out at 23 °C at a scattering angle of 90°.

3. Results

3.1. Physicochemical characterization

Nanosomal Amphotericin B was prepared using soy phosphatidylcholine and sodium cholesteryl sulfate as excipients. No organic solvents or detergents were used during the entire preparation of Nanosomal Amphotericin B. The exact size and distribution of Nanosomal Amphotericin B preparation was determined using the standard method of dynamic light scattering as shown in Fig. 1. The mean diameter of Nanosomal Amphotericin B preparation was found to be 40.2 nm after reconstitution. Electron microscopy of Nanosomal Amphotericin B after freeze-fracture revealed the presence of a homogenous population of nanosized particles having an average size less than 100 nm (Fig. 2). The reconstituted Nanosomal Amphotericin B suspension was found to be stable for up to 48 h at room temperature. Further dilution of reconstituted form was conducted with 5% dextrose. The diluted suspension was also found to be stable for 48 h at both 2–8 °C and room temperature as content of Nanosomal Amphotericin B confirmed by HPLC analysis. In both cases, the mean particle size remained below 100 nm (Table 1).

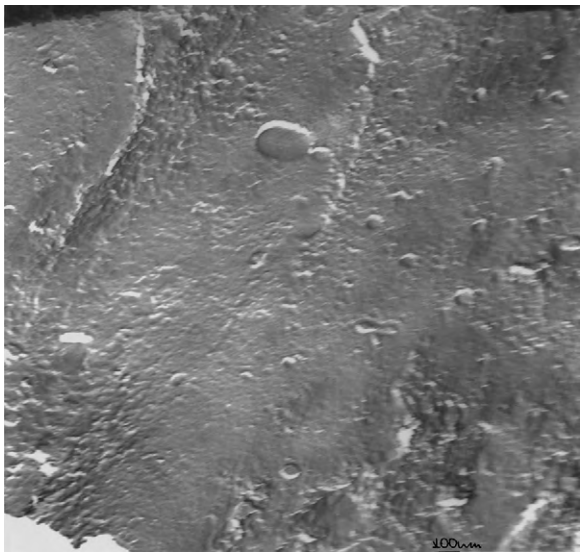

Fig. 2. Freeze-fracture electron micrograph of Nanosomal Amphotericin B. The replica was examined at a JEOL 100 CX TEM/Microanalytical Laboratories, Inc. and at a JEOL1230 TEM/Stanford University.

Table 1
Analysis of Nanosomal Amphotericin B.

Test	Initial reconstitution[a] (±SD)	48 h after reconstitution[a] (±SD)	Dilution with 5% dextrose[b] (±SD)
Mean particle size, nm	34.6 (±3.8)	49.5 (±26.3)	42.9 (±1.4)
Amphotericin B, % (HPLC)	104.7 (±2.1)	107.9 (±0.7)	104.5 (±1.9)
pH	5.65 (±0.16)	6.02 (±0.01)	5.91 (±0.04)

[a] Samples were reconstituted with water at a concentration of 2 mg/mL and stored for 48 h at 23–25° C before analysis.
[b] Sample was diluted to a concentration of 0.1 mg/mL.

3.2. Animal toxicology

Rabbits were administered intravenously once daily for 28 days with Nanosomal Amphotericin B. During the dosing period and at termination, the quantity of food consumed by animals in different dose groups was found to be comparable with that of control animals. Rabbits were treated with Nanosomal Amphotericin B at 1, 2.5 and 5 mg/kg. All the animals survived after 28-day treatment period and even at the highest dose no mortality was observed. In addition, animals from all groups including the highest dose of Nanosomal Amphotericin B showed no significant loss of body weight. Hematological investigations revealed no significant changes in the values of hematological parameters compared to controls at all the dose level tested (Table 2). The values obtained were either within normal laboratory limits suggesting no dose dependent effect. The bone marrow examination also did not reveal any abnormality.

Biochemical analysis revealed normal levels of blood urea nitrogen (BUN) and creatinine in male and female rabbits at 1 and 2.5 mg/kg dose levels (Table 3). However, rabbits treated with the test formulation at 5 mg/kg showed elevated BUN levels in male and female animals and significant elevated creatinine levels only in female animals. Organ weight data of male rabbits from control and different dose groups was found to be comparable. However, female rabbits at 5 mg/kg revealed increased average relative weights of spleen to 56.37 compared to 41.63 in the untreated animals. Gross pathological examination did not reveal any abnormality attributable to the treatment in rabbits. Histopathological examination revealed minimal to mild greenish yellow deposits in liver and minimal to moderate greenish yellow deposits in spleen. It is suggestive of accumulation of test substance or its metabolites in liver and spleen with no reaction within the tissue or associated signs of hepatotoxicity. It is known that Amphotericin B products get accumulated in the liver and spleen (Amphocil™/Amphotec®, Patient Information leaflet, 2005; Ambisome®, Package insert, 2008).

3.3. RBC hemolysis

To examine toxicity towards human RBCs, Amphotericin B formulations were evaluated in terms of their ability to induce hemolysis. In PBS control or Nanosomal placebo (Drug free formulation) resulted in no hemolysis. The hemolytic activity of Amphotericin B formulations was compared with RBCs hemolysis in the presence of water. The rate of hemolysis with Amphotericin B deoxycholate was significantly higher compared to Liposomal Amphotericin B (Ambisome®) or Nanosomal Amphotericin B (Fig. 3). Moreover, the hemolysis with Nanosomal Amphotericin B was slightly lower compared to Ambisome® at the tested concentration range (10–200 μg/mL).

3.4. Efficacy of Nanosomal Amphotericin B and liposomal (Ambisome®) Amphotericin B

The survival of Aspergillus fumigatus infected mice following intravenous administration of 5% dextrose (control), Nanosomal Amphotericin B or Ambisome® was monitored during the 5 days of the dosing period and additional 5 days for the observation period, a total of 10 days. The result (Fig. 4) indicates that all the 10 infected mice in the control group treated with 5% dextrose solution died within 5 days due to fungal infection. These mice showed signs of fungal infection after 24 h and 90% of the mortality occurred between day 2 and 4. The mortality in the Ambisome® treated mice was delayed up to day 4 but only 30% mice survived by day 10 (Fig. 4) indicating an improved effect on mortality by Ambisome®. However, 90% of the mice treated with Nanosomal Amphotericin B survived until day 10. The results indicate a 30% survival against the fungal infection by Ambisome® and 90% survival by Nanosomal Amphotericin B (Fig. 4). Thus, the Nanosomal Amphotericin B had the greater therapeutic effect compared to Ambisome® at the same dose level in controlling Aspergillosis in experimental mice.

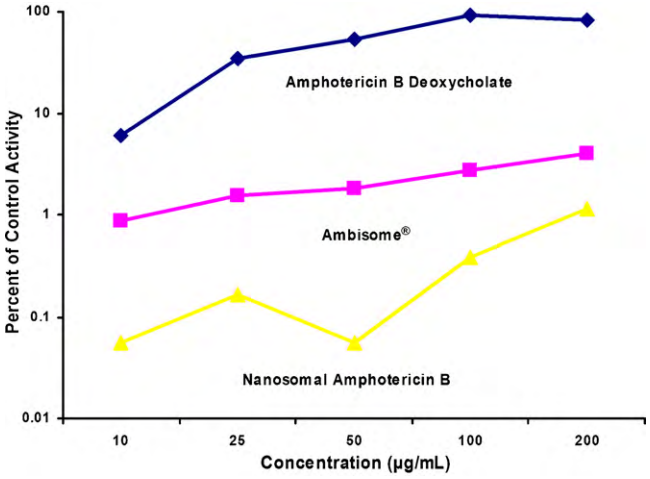

Fig. 3. Effect of Amphotericin B formulations on human RBCs. The Amphotericin B formulations at indicated concentrations were incubated with freshly processed human RBCs. The microtiter plate containing samples was constantly agitated for 1 h at 37 °C. The plate was centrifuged at 3000 rpm for 10 min and an aliquot of supernatant was taken to measure the hemoglobin content at 550 nm on a SpectraMax M2.

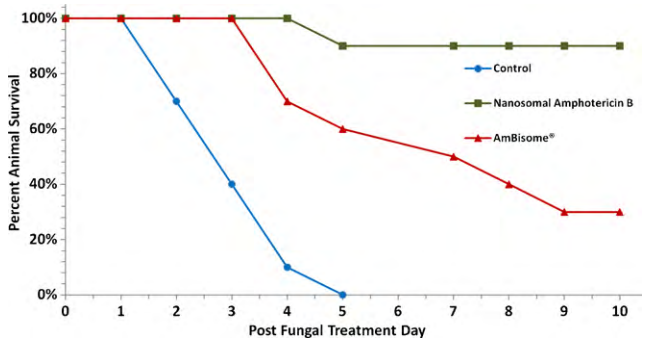

Fig. 4. Percent survival of Aspergillus fumigatus pretreated mice following daily intravenous administrations of 5% dextrose solution (control), and Nanosomal Amphotericin B, and Ambisome® (Liposomal Amphotericin B) at the dose rate of 2 mg Amphotericin B/kg body weight for 5 consecutive days.

Table 2
Blood chemistry profile in Nanosomal Amphotericin B treated male and female rabbits.

Group no.	Dose (mg/kg)		Hb (g %)	Total RBC ($\times 10^6/\mu L$)	Rt (%)	HCT (%)	Platelets ($\times 10^5/\mu L$)	Total WBC ($\times 10^5/\mu L$)
Male rabbits								
I	Control	Mean	16.20	7.34	3.83	46.97	270.33	5.37
		±SD	3.47	1.39	0.35	9.68	81.14	1.32
II	1	Mean	16.90	7.31	3.90	48.43	294.00	5.37
		±SD	0.44	0.17	0.26	0.99	55.33	0.74
III	2.5	Mean	13.93	6.25	3.87	40.30	254.67	4.47
		±SD	0.67	0.64	0.47	3.03	22.03	0.72
IV	5	Mean	16.00	7.06	4.03	45.67	177.67	6.23
		±SD	1.67	0.77	0.15	4.38	67.60	1.88
Female rabbits								
I	Control	Mean	15.00	6.78	3.90	43.13	392.67	4.03
		±SD	2.33	1.05	0.66	6.69	96.93	1.72
II	1	Mean	14.93	6.84	3.83	45.57	373.67	3.43
		±SD	1.97	1.08	0.50	7.37	13.05	1.70
III	2.5	Mean	14.07	6.44	3.87	41.13	310.33	5.33
		±SD	0.46	0.40	0.70	1.69	4.73	0.06
IV	5	Mean	15.37	6.84	3.93	44.07	349.33	5.40
		±SD	0.92	0.39	0.74	2.78	18.82	0.92

4. Discussion

A well-characterized organic solvent and detergent free homogeneous formulation of Amphotericin B is developed using naturally occurring lipids, which are categorized as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration. Nanosomal Amphotericin B formulation is prepared by mixing Amphotericin B and lipids that are directly homogenized to obtain nanosized particles. The advantage of this new formulation includes ease of preparation and reduction in the production cost. It has been described earlier that small size particles help in longer circulation time and slower uptake in the reticuloendothelial system. This helps in the availability of the drug for longer time in plasma and sustains delivery of drug to the site of infection (Storm and Woodle, 1998).

Amphotericin B formulations including those with the lipid carriers have shown low levels of haematocrit (Larabi et al., 2004) whereas rabbits treated with Nanosomal Amphotericin B for 28 days did not show any decrease in haematocrit values. In addition, bone marrow examination did not reveal any abnormality after treatment with Nanosomal Amphotericin B. These results indicate that Nanosomal Amphotericin B causes less hemotoxicity.

Amphotericin B deoxycholate and other lipid-based formulations such as Abelcet® and Ambisome® induce increase in the hepatotoxicity leading to the elevated levels of serum AST and ALT transaminases (Ambisome®, Package insert, 2008; Larabi et al., 2004). This could be due to the composition and proportion of the lipid associated with Amphotericin B. In contrast, administration of Nanosomal Amphotericin B in rabbits (Table 3), even at a highest dose did not show any significant increase in the serum AST and ALT transaminases levels indicating less accumulation of the Amphotericin B in the liver.

Nanosomal Amphotericin B was also found to be significantly less hemolytic against human RBCs than deoxycholate form (Fungizone) but comparable to Ambisome®. Treatment of infected mice with Nanosomal Amphotericin B resulted in 90% survival compared to 0% survival in the control. Infected mice treated with Ambisome® at equal doses and same schedule like Nanosomal Amphotericin B resulted in only 30% survival of mice. These efficacy results demonstrate a therapeutic superiority of Nanosomal Amphotericin B over the Ambisome®. However, no formulations have been shown to have a better therapeutic profile compare to Fungizone (Brajtburg and Bolard, 1996).

In conclusion, a new Nanosomal Amphotericin B formulation has been developed without using organic solvents during the entire manufacturing process, having an average particle size less than 100 nm. The Nanosomal Amphotericin B formulation is stable, less toxic and provides greater protection in mice against fungal

Table 3
Biochemical analysis of male and female rabbits blood samples after day 29.

Group no.	Dose (mg/kg)		Total protein (g%)	BUN (mg %)	Creatinine (mg %)	ALT (IU/L)	AST (IU/L)	AP (IU/L)	Blood glucose (mg %)
Male rabbits									
I	Control	Mean	7.11	38.67	0.96	39.33	67.00	72.67	136.67
		±SD	0.20	5.51	0.04	6.43	3.46	0.58	6.43
II	1	Mean	7.43	35.67	0.95	38.00	65.33	73.00	141.33
		±SD	0.57	2.89	0.06	7.94	6.35	9.64	4.93
III	2.5	Mean	7.60	39.00	0.98	42.00	65.00	75.33	135.00
		±SD	0.62	4.36	0.02	1.00	4.58	2.89	7.21
IV	5	Mean	7.82	68.00[a]	1.45	34.67	59.33	70.67	136.67
		±SD	0.71	1.73	0.44	1.53	3.79	7.77	9.29
Female rabbits									
I	Control	Mean	7.36	34.33	0.94	41.33	59.33	76.33	132.67
		±SD	0.45	4.51	0.11	0.58	6.03	3.06	4.73
II	1	Mean	7.63	33.33	1.00	41.00	63.00	67.67	143.33
		±SD	0.32	3.06	0.10	3.61	4.36	6.11	4.73
III	2.5	Mean	7.77	30.00	0.94	36.67	64.33	74.67	137.33
		±SD	0.18	1.00	0.05	6.35	3.51	4.62	2.89
IV	5	Mean	7.29	60.33[a]	1.7[a]	40.00	62.00	76.67	135.33
		±SD	0.38	5.86	0.22	6.24	12.12	4.93	3.21

[a] Significant at 99% level of confidence ($P < 0.01$).

infection compared to the partial protection by Ambisome®. Studies in humans are underway to evaluate therapeutic benefit of Nanosomal Amphotericin B.

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