Toxicity, stability and pharmacokinetics of amphotericin B in immunomodulator tuftsin-bearing liposomes in a murine model

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Objectives: In the present study we evaluated the pharmacokinetics and toxicity of amphotericin B in immunomodulator tuftsin-loaded liposomes in a murine model.

Methods: Stability of amphotericin B liposomes was tested by incubating one volume of liposomal formulations of amphotericin B with nine volumes of serum. The pharmacokinetics of amphotericin B in Candida albicans-infected mice treated with conventional and tuftsin-loaded amphotericin B liposomes was evaluated over a period of 24 h. In vitro toxicity of amphotericin B deoxycholate, as well as amphotericin B liposomes, was tested by incubation with human erythrocytes for 1 h at 37°C. To assess amphotericin B-induced in vivo toxicity, BALB/c mice were injected with three doses of amphotericin B deoxycholate, as well as amphotericin B liposomal formulations on days 1, 2 and 3 post C. albicans infection. Blood from treated mice was taken by retro-orbital puncture to test renal function parameters such as serum creatinine and urea.

Results: In vitro stability studies revealed that tuftsin-bearing amphotericin B liposomes released only 11% of the total liposomal amphotericin B in the serum, while it was found to be 19% from identical tuftsin-free amphotericin B liposomes. Both tuftsin-loaded as well as tuftsin-free liposomal formulations of amphotericin B induced ~20% haemolysis of erythrocytes at a dose of 40 mg/L, while the same amount of drug in amphotericin B deoxycholate caused 100% lysis of the erythrocytes. Pharmacokinetic studies revealed that subsequent to administration of various formulations of amphotericin B, there was 32 mg/L amphotericin B in the systemic circulation of mice treated with tuftsin-bearing amphotericin B liposomes, while it was 25 mg/L for amphotericin B liposomes, 4 h post drug administration. In vivo toxicity studies demonstrated that the amphotericin B deoxycholate formulation induced elevations in serum creatinine (~300% of control) and blood urea (~380% of control) values, while these values were substantially less (blood urea ~150% of control and serum creatinine ~210% of control) in the animals treated with the tuftsin-loaded amphotericin B liposomal formulation. Further, the administration of amphotericin B deoxycholate (1 mg/kg) in BALB/c mice at a dose of 1 mg/kg body weight led to the accumulation of 18.6 ± 5.25 g/kg (of amphotericin B) in kidneys. On the other hand, administration of liposomal amphotericin B and tuftsin-bearing liposomal amphotericin B at a dose of 5 mg/kg body weight resulted in accumulation of 8.8 ± 2.0 and 4.0 ± 1.6 g/kg of amphotericin B, respectively, in the kidneys of treated animals.

Conclusions: Co-administration of immunomodulator tuftsin along with liposomal formulations of amphotericin B successfully minimizes toxicity, as well as other side effects of the drug. Interestingly, tuftsin also increased the stability of liposomal amphotericin B. Superior efficacy, reliable safety and favourable pharmacodynamics of tuftsin-loaded amphotericin B liposomes suggest their potential therapeutic value in the management of fungal infections.

Keywords: antifungal agents, C. albicans, renal function tests, erythrocytes

Introduction

Extensive and frequent use of fluconazole for treatment of common fungal infections has resulted in the emergence of azole-resistant strains of pathogenic fungi.1–4 Amphotericin B has been used as the ‘gold standard’ antifungal drug since 1960.5,6 The use of lipid formulations of amphotericin B has made the drug more tolerable, but they have not shown remarkable...
Tuftsin is a tetrapeptide immunomodulator spanning 289–292 amino acid residues (Thr-Lys-Pro-Arg) of the IgG molecule. Tuftsin augments immune functions of immune cells, primarily macrophages, neutrophils and monocytes. Tuftsin has been found to increase macrophage-mediated phagocytosis, splenocyte proliferation, bactericidal and tumoricidal activities, and release of certain cytokines such as interleukin-1 and tumour necrosis factor-α. Tuftsin-deficient patients have been found to be more susceptible to bacterial infections. Taking into consideration the high occurrence of fungal infections in immunocompromised persons, we formulated immunomodulator tuftsin-bearing amphotericin B liposomes. This formulation, like conventional amphotericin B liposomes, not only decreases the toxicity of amphotericin B, but also activates the front-line immune cells such as macrophages and neutrophils to fight against fungal pathogens. Our previous studies showed tuftsin-mediated augmentation of the efficacy of amphotericin B and nystatin liposomes against drug-resistant, as well as drug-susceptible, isolates of Candida albicans and Aspergillus fumigatus in a murine model. Moreover, tuftsin has been shown to play an important role in the early recovery of the depleted leucocytes in cyclophosphamide-treated mice as well.

In order to exploit tuftsin-bearing amphotericin B liposomes as a potential candidate formulation for treatment of opportunistic fungal infection, it is always desirable to perform extensive pharmacodynamic studies. In the present study, we investigated both in vivo and in vitro toxicity of tuftsin-loaded amphotericin B liposomes and compared it with amphotericin B deoxycholate and amphotericin B liposomes without tuftsin. Besides, we performed single dose pharmacokinetics and a tissue distribution study of amphotericin B in C. albicans-infected BALB/c mice.

### Materials and methods

#### Materials

Cholesterol was bought from Centron Research Laboratory (Bombay, India) and was used after crystallization with methanol. Egg phosphatidylcholine (egg PC) was isolated and purified as described previously. Human blood for isolation of serum was obtained from the blood bank of J. N. Medical College, Aligarh, India. Amphotericin B was purchased from Sigma Chemical Co. (St Louis, MO, USA). Tetrapeptide tuftsin was synthesized followed by its modification by adding a long fatty acyl chain according to a published method.

#### Animals

Female BALB/c mice of average weight 24 g were purchased from JALMA Leprosy Research Institute, Agra, India. The animals were given a standard pellet diet (Hindustan Lever Ltd) and water ad libitum. Mice were checked daily for their mortality and morbidity prior to commencement of the study and only healthy mice were included in the study. The techniques used for bleeding, injection and sacrifice of animals were strictly performed following mandates approved by the Animal Ethics Committee [Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India].

#### Strain

C. albicans was a gift from Professor A. Malik, Microbiology Department, JNMC, Aligarh. It was isolated from a patient suffering from oropharyngeal candidiasis and was maintained both in vitro and in the animals. The identity of the strain was confirmed by biochemical and microbiological procedures.

#### Antifungal susceptibility testing

The MIC of amphotericin B for the C. albicans strain was determined by the broth macrodilution method described by the NCCLS. Amphotericin was tested over a final concentration range of 0.02–2 mg/L. Testing was performed in 96-well round-bottomed microtitre plates. A cell suspension of C. albicans was prepared in RPMI 1640 medium and adjusted to give a final inoculum concentration of 2 × 10⁵ cells/mL. The wells containing fungal inoculum with different concentrations of the drug and proper controls were incubated for 48 h. The MIC was defined as the lowest concentration of the drug at which there was complete inhibition of the growth. The MIC of amphotericin B used in the present study was found to be 0.25 mg/L.

#### Infection model

Each mouse was infected with C. albicans (1 × 10⁶ cfu/mouse) by intravenous route. Mice were made leucopenic by injecting cyclophosphamide (250 mg/kg) via lateral tail veins 2 days before C. albicans infection. C. albicans strain obtained from JNMC was subcultured on Sabouraud dextrose agar plates and the inoculum was prepared by placing some fresh colonies in 5 mL of sterile 0.9% saline. Numbers of cells were counted using a haemocytometer.

#### Preparation of liposomes

Liposomes were prepared by mixing egg PC (49 µmol) and cholesterol (21 µmol) without or with modified tuftsin (7–8% by PC weight) by a sonication method. All the ingredients, along with amphotericin B (drug/lipid, 1:20 molar ratio), were dissolved in a round-bottomed flask in chloroform/methanol (1:1, v/v). The solvents were carefully evaporated under reduced pressure to form a thin lipid film on the wall of the flask. The lipid film was hydrated with 2.0 mL of 150 mM sterile saline solution followed by sonication (1 h, 4°C) in a bath-type sonicator. The sonicated preparation was centrifuged at 10 000 g for 1 h at 4°C to remove traces of un-dispersed lipid and finally dialysed against normal saline for 24 h at 4°C in the dark.

#### Determination of size, lamellarity and structure of liposomes

Liposome lamellarity, size and structure were evaluated by transmission electron microscopy. Briefly, a drop of liposomal formulation...
Stability and toxicity of tuftsin-containing amphotericin B liposomes

was applied with a micropipette in a pioloform-coated copper grid, held by a fine watchmaker’s tweezer attached to a fixed stand. The drop was left on the grid for 10–15 min. The drop was then touched with the edge of a filter paper and 10–15 drops of the negative stain (2% potassium phosphotungstic acid at pH 7.0) were flushed over the grid (the filter paper was kept as a drain). The drop was partially dried with the filter paper, in order to leave a thin fluid film that was allowed to dry up. The grid was viewed in a Tecnai 12 Biotwin transmission electron microscope (Hillsboro, OR, USA) using 120 kV. The negatively stained images were acquired using a Gatan Digital camera (Pleasanton, CA, USA). The size of the liposomes was found to be in the range of 50–100 nm. The majority of the liposomes were found to be unilamellar, but some of them were multimamellar.

Estimation of liposome-intercalated amphotericin B and tuftsin

The intercalation efficiency of amphotericin B in liposomes was estimated by the comparative release of amphotericin B in our laboratory.11 The amount of amphotericin B incorporated in liposomes was estimated by using a HPLC method as well. Briefly, the sample (20 μL) was injected onto a Hypersil octyldecyl-silane 5 μm particle size analytical column [150 by 4.6 mm (internal diameter)]. Detection was accomplished with a UV-visible-light detector set at 405 nm. A standard curve of amphotericin B was plotted using calibrating peak area versus amount of the drug injected into the column. The intercalation efficiency of amphotericin B in both plain egg PC and tuftsin-bearing liposomes was found out to be of the same order as estimated by spectrophotometric method (90 ± 4%).

To enhance the binding of liposomes to leucocytes, tuftsin was modified at the C terminus by attaching a long fatty acyl residue through an ethylenediamine spacer arm [Thr-Lys-Pro-Arg-NH-(CH$_2$)$_2$-NH-COCH$_3$] according to the published procedure. The tuftsin entrapped in the drug-containing liposomes was estimated by bicinchoninic acid (BCA) method as modified in our laboratory. Briefly, the liposomes (given volume) were lysed with 10% Triton X-100 solution (the final concentration of Triton X-100 was maintained at 1%). The mixture of solutions A (containing sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide) and B (4% cupric sulphate) of BCA reagent was added to an aliquot of tuftsin-incorporated liposomes digested with Triton X-100. The reaction mixture was incubated at 37°C for 45 min. The absorbance was measured at 600 nm and the content of tuftsin associated with liposomes was determined using a standard curve of tuftsin plotted in the presence of Triton X-100. The intercalation efficiency of amphotericin B in liposomes was estimated by dissolving the amphotericin B obtained at 15 min was used as the maximum concentration of amphotericin B obtained at 15 min was used as the maximum concentration ($C_{max}$).

For the tissue distribution study, the concentration of amphotericin B was determined in the liver and kidneys of the infected mice after treatment with amphotericin B deoxycholate, as well as liposomal formulations of amphotericin B by intraperitoneal route. Animals were treated with amphotericin B deoxycholate (5 mg/kg) and tuftsin-free or tuftsin-loaded liposomal amphotericin B (5 mg/kg). Livers and kidneys were taken out from the treated mice 12 h after drug administration. Tissues were rinsed with chilled saline for further analysis.

Processing of blood and tissues for HPLC analysis

Samples were processed for the separation of amphotericin B according to the published procedure. An equal volume (100 μL) of the serum and chilled HPLC grade acetonitrile were mixed and incubated for 5–10 min. The mixture was vortexed for 30 s followed by centrifugation. The clear supernatant was separated and filtered through a micropore filter before being submitted to an assay.

For tissue processing, weighed portions of organs were washed in phosphate-buffered saline (PBS) (20 mM, pH 7.4). Samples were then reweighed and homogenized with ice-cold HPLC grade methanol in a high-speed tissue homogenizer in an ice bucket. Standards and control samples of the tissues for the drug analysis were prepared in the same manner by homogenizing normal tissues in HPLC grade methanol by adding known amounts of amphotericin B. Homogenized samples were centrifuged at 1500 g for 10 min and resulting supernatants were transferred for filtration through a micropore filter.

HPLC analysis of each sample was performed in duplicate using a reverse-phase Bondapack column. The mobile phase consisted of 0.005 M EDTA buffer and methanol (35:65, v/v) and the flow rate was kept at 1.2 mL/min. The results were quantified by comparison of amphotericin B peak at 405 nm with a standard of known concentration eluted at the same retention time. The limit of detection was 0.1 mg/L.

Determination of in vitro toxicity of amphotericin B deoxycholate and liposomal amphotericin B to human erythrocytes

Human blood collected in ACD solution (acid citrate dextrose) was centrifuged (1500 g, 15 min) and plasma was separated out. The red blood cells (RBCs) were washed with isotonic PBS (20 mM, pH 7.4)
three times by centrifugation. The erythrocytes were diluted with the same isotonic buffer and 1% haematoцит was prepared for experimental use. Amphotericin B (free as well as liposomal form) was added at varying concentrations (5–50 mg/L) to the haematoцит. To study haemolysis, a solution of RBCs was incubated with 1 mL of amphotericin B deoxycholate and both formulations of amphotericin B liposomes at 37°C for 1 h. After incubation, the reaction mixture was centrifuged at 1500 g and the supernatant was collected and analysed for haemoglobin by UV-visible spectroscopy at 576 nm. The amount of haemoglobin released by 0.1% Triton X-100-treated erythrocytes was considered as a control of 100% lysis.

Assessment of in vivo renal toxicity
Renal toxicity was monitored by applying a multidose regimen to determine biochemical profiles of blood urea and serum creatinine. Each mouse was infected with 10⁵ cells of C. albicans in a 0.2 mL volume of normal saline through the lateral tail vein. The treatment with amphotericin B was given via intravenous route on days 1, 2 and 3 post C. albicans infection. The dose of amphotericin B given to the infected animals was as follows: amphotericin B deoxycholate, 1 mg/kg; liposomal amphotericin B, 5 mg/kg; and tuftsin-bearing liposomal amphotericin B, 5 mg/kg. The blood was collected from three mice of each group before the first dose and after the last dose of the drug administration. The blood was allowed to clot at room temperature and serum was separated for investigation.

There were 10 mice in each group and groups were divided as follows:

(i) Immunocompetent
   (a) Control
   (b) Treated with amphotericin B deoxycholate
   (c) Treated with liposomal amphotericin B
   (d) Treated with tuftsin-bearing liposomal amphotericin B

(ii) Mice were made leucopenic by injecting cyclophosphamide (250 mg/kg) by lateral tail vein 2 days before C. albicans infection and were divided as follows:
   (a) Control
   (b) Treated with amphotericin B deoxycholate
   (c) Treated with liposomal amphotericin B
   (d) Treated with tuftsin-bearing liposomal amphotericin B

Acute toxicity of amphotericin B deoxycholate and liposomal formulations of amphotericin B in C. albicans-infected mice
To assess the acute toxicity of tuftsin-loaded amphotericin liposomes, amphotericin B deoxycholate (1–3 mg/kg) and liposomal formulations of amphotericin B (5–25 mg/kg) with or without tuftsin were administered in increasing doses to C. albicans-infected mice. Treatment was started 12 h after intravenous infection with C. albicans (1 × 10⁶ cfu/mouse in 100 µL of PBS). Acute toxicity was defined as death of mice within 3 h of drug administration.

Statistics
Statistical analysis among various groups was performed by one-way ANOVA followed by Bonferroni test using prism software, version 4.0. The pharmacokinetics study was compared by two-tailed paired t-test. A P value <0.05 was considered significant.

Results
Release of liposomal amphotericin B in human serum
The incubation of the liposomal formulation of amphotericin B with human serum resulted in slow release of the drug. Tuftsin-loaded amphotericin B liposomes were found to exhibit more stability in human serum compared with amphotericin B entrapped in the same liposomes without tuftsin. There was 11% leakage of the drug out of total amphotericin B incorporated in tuftsin-bearing amphotericin B liposomes into the serum, while it was found to be 19% in the case of amphotericin B liposomes without tuftsin (Figure 1).

Pharmacokinetics and tissue distribution of amphotericin B by HPLC and spectrophotometry
The pharmacokinetics of amphotericin B was studied in the serum of C. albicans-infected immunocompetent, as well as leucopenic, mice following intravenous single doses of liposomal amphotericin B formulations as described in the Materials and methods section. The serum concentration of the drug was found to be higher in mice treated with tuftsin-loaded amphotericin B liposomes compared with those treated with identical amphotericin B liposomes without tuftsin (Figure 2). Leucopenic mice also showed similar pharmacokinetics with a slightly higher concentration of the drug in the systemic circulation (data not shown).

Administration of tuftsin-free and tuftsin-loaded liposomal amphotericin B at a single dose of 5 mg/kg resulted in Cmax of amphotericin B of 52.6 and 64.2 mg/L 15 min after drug administration. There were no significant differences in AUC0–24 for tuftsin-free (40.2 mg·h/L) and tuftsin-loaded (44.2 mg·h/L) liposomes. Tuftsin-loaded liposomal amphotericin B showed a decrease in clearance and increased half-life (CL = 0.40 L/h, t1/2 = 3.6 h) compared with tuftsin-free amphotericin B liposomes (CL = 0.34 L/h, t1/2 = 2.8 h). The tuftsin-loaded liposomal formulation restricted the distribution of the drug in comparison with liposomal amphotericin B without tuftsin, as shown by the volume of distribution (V; 56 versus 68 mL/kg of the body weight).

In tissue distribution studies, the animals were sacrificed 12 h after drug administration. Liver and kidneys were taken out and homogenized for extraction of the drug. The extracted amphotericin B was analysed by HPLC, and animals treated with tuftsin-loaded amphotericin B liposomes had higher concentrations of the drug in liver and lower concentrations in kidneys compared with mice treated with amphotericin B deoxycholate (Table 1).

Incorporation of amphotericin B in tuftsin-loaded liposomes markedly reduces their toxicity to erythrocytes
Among various formulations used in the present study, amphotericin B deoxycholate was found to show maximum toxicity to erythrocytes as evident from drug-induced haemolysis of human erythrocytes in a dose-dependent manner. The incorporation of amphotericin B in conventional as well as tuftsin-loaded liposomes resulted in diminution of its toxic property to a surprisingly low level. Amphotericin B in tuftsin-load liposomes showed less toxicity to erythrocytes than amphotericin B deoxycholate. Amphotericin B deoxycholate at a dose of 35 mg/L causes 100% haemolysis of erythrocytes, while liposomal amphotericin B at the same dose causes only 20% haemolysis (Figure 3).
Toxicity in animal models

Treatment with amphotericin B liposomal formulations was found to be associated with less nephrotoxicity compared with treatment with amphotericin B deoxycholate. The level of serum creatinine and blood urea increased (~2-fold over control values) in mice treated with amphotericin B deoxycholate (Figure 4), while mice treated with tuftsin-free or tuftsin-loaded amphotericin B liposomes showed less increase in renal function parameters. This renal toxicity was more pronounced (about 3-fold over control values) in leucopenic mice upon their exposure to amphotericin B (Figure 4).

Acute toxicity results of various formulations of amphotericin B, namely amphotericin B deoxycholate and tuftsin-free or tuftsin-loaded amphotericin B liposomes, in mice showed highly diminished toxicity of the drug upon incorporation in liposomes.

Discussion

The results of the present investigation demonstrated a major reduction in the toxicity of amphotericin B upon incorporation in liposomes compared with amphotericin B deoxycholate. Among various antifungal agents, polyene antibiotics have been supposed to be highly toxic to the host cells. To evaluate the toxicity of liposomal amphotericin B, with or without immuno-modulator tuftsin, erythrocytes were incubated with both amphotericin B deoxycholate as well as liposomal formulations of amphotericin B. We found that amphotericin B incorporated in

Table 1. Concentration of amphotericin B (Amp B) in liver and kidneys of mice

<table>
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<tr>
<th>Group</th>
<th>Liver (mg/kg)</th>
<th>Kidneys (mg/kg)</th>
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<tbody>
<tr>
<td>Amp B deoxycholate (5 mg/kg)</td>
<td>24.4 ± 6.8</td>
<td>18.6 ± 5.25</td>
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<tr>
<td>Liposomal Amp B (5 mg/kg)</td>
<td>54.0 ± 9.6</td>
<td>8.8 ± 2.0</td>
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<tr>
<td>Tuftsin-bearing liposomal Amp B</td>
<td>59.2 ± 6.4</td>
<td>4.0 ± 1.6</td>
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<td>Amp B (5 mg/kg)</td>
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Amp B deoxycholate versus liposomal Amp B, P = 0.0018; Amp B deoxycholate versus tuftsin-bearing liposomal Amp B, P = 0.0017; liposomal Amp B versus tuftsin-bearing liposomal Amp B, P = 0.3488.
tuftsin-free, as well as tuftsin-loaded, liposomes induced less haemolysis in comparison with amphotericin B deoxycholate. The observed reduced toxicity of tuftsin-loaded amphotericin B liposomes like conventional amphotericin B liposomes would allow accommodation of much higher doses of the drug for in vivo use. This result is also supported by in vivo results of acute toxicity of amphotericin B formulations. Mice treated with liposomal formulations of amphotericin B showed a maximum tolerated dose that was 5-fold greater than amphotericin B deoxycholate. This would certainly be advantageous to treat less susceptible or drug-resistant isolates of fungal pathogens, which do not respond to lower drug doses of antifungal agents.

Amphotericin B deoxycholate (1 mg/kg), tuftsin-bearing liposomal amphotericin B (5 mg/kg) and liposomal amphotericin B (5 mg/kg) were injected into C. albicans-infected mice on days 1, 2 and 3 post-infection. The renal toxicity was assessed by estimating serum creatinine and blood urea in mice. We found that the levels of serum creatinine and blood urea were elevated in mice treated with amphotericin B deoxycholate and this increase was found to be more in leucopenic mice. The enhanced renal toxicity in leucopenic mice may be the result of the cumulative effect of cyclophosphamide plus amphotericin B therapy. Amphotericin B in free form has been found to bind with low-density lipoproteins (LDL) and there are high numbers of

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**Figure 4.** (a) Serum creatinine and blood urea values of amphotericin B-treated C. albicans-infected immunocompetent BALB/c mice. C. albicans-infected mice were treated with amphotericin B deoxycholate (1 mg/kg) or liposomal amphotericin B formulations (5 mg/kg) by intravenous route on days 1, 2 and 3 post-infection as described in the Materials and methods section. Blood from three mice was taken and serum creatinine values were determined. Amphotericin B deoxycholate (Amp B deoxycholate) versus liposomal amphotericin B (Lip-Amp B), P < 0.001; Amp B deoxycholate versus tuftsin-bearing liposomal amphotericin B (Tuft-lip-Amp B), P < 0.001; Lip-Amp B versus Tuft-lip-Amp B, P > 0.05. (b) Blood urea values of amphotericin B deoxycholate-treated C. albicans-infected immunocompetent BALB/c mice. Amp B deoxycholate versus Lip-Amp B, P < 0.001; Amp B deoxycholate versus Tuft-lip-Amp B, P < 0.001; Lip-Amp B versus Tuft-lip-Amp B, P > 0.05. (c) Serum creatinine values of amphotericin B deoxycholate-treated C. albicans-infected leucopenic BALB/c mice. Leucopenic mice were treated with amphotericin B deoxycholate (1 mg/kg) or liposomal amphotericin B (5 mg/kg) on days 1, 2 and 3 post C. albicans infection by intravenous route as described in the Materials and methods section. Amp B deoxycholate versus Lip-Amp B, P < 0.01; Amp B deoxycholate versus Tuft-lip-Amp B, P < 0.01; Lip-Amp B versus Tuft-lip-Amp B, P > 0.05. (d) Blood urea values of amphotericin B deoxycholate-treated C. albicans-infected leucopenic BALB/c mice. Amp B deoxycholate versus Lip-Amp B, P < 0.001; Amp B deoxycholate versus Tuft-lip-Amp B, P < 0.001; Lip-Amp B versus Tuft-lip-Amp B, P > 0.05.
renal LDL receptors that mediate the cellular uptake of amphotericin B. This interaction of amphotericin B, LDL and renal receptors is believed to be a major contributor to the dose-limiting nephrotoxicity of amphotericin B. Low renal toxicity of amphotericin B liposomes is supported by the results of drug distribution studies that showed reduced distribution of amphotericin B in kidneys of mice treated with tuftsin-free or tuftsin-loaded amphotericin B liposomes. In fact, the data in the present study show that a major fraction of the drug is found in liver, the organ rich in reticuloendothelial system (RES), when the drug is administered as a liposomal formulation. The liposome-mediated passive targeting of amphotericin B enables the drug to be accumulated in these organs. This may be explained by the fact that lipid formulations of amphotericin B have a high affinity for binding to high-density lipoproteins (HDL). Binding to HDL promotes uptakes in RES, which has a relatively high level of expression of HDL receptors. In contrast, the free drug has high affinity for LDL. The preferential uptake of liposomal amphotericin B by hepatic and splenic macrophages leads to reduced availability of the drug to kidneys and other organs, which ultimately helps in reducing nephrotoxicity and improving therapeutic index compared with free drug.

The stability of tuftsin-free and tuftsin-loaded amphotericin B liposomes was monitored both in vitro and in vivo. Amphotericin B liposomes upon incubation with human serum release entrapped drug into the serum. The increased stability of tuftsin-loaded amphotericin B liposomes in the serum is observed due to the presence of tuftsin in the liposomal bilayer, as when amphotericin B liposomes without tuftsin were incubated with the serum, the liposomal structure was relatively lost. Tuftsin seems to stabilize the bilayer structure of the liposomes; it seems it is protecting the liposomes from the action of serum proteins. A very low percentage of amphotericin B (11%) is released from tuftsin-bearing liposomal amphotericin B in vitro during 24 h of incubation with 90% (v/v) serum at 37°C. The pharmacokinetic study clearly showed a prolonged stay of amphotericin B in the systemic circulation of tuftsin-bearing liposomal amphotericin B-treated animals compared with those treated with amphotericin B liposomes without tuftsin. This may also be one of the reasons for the observed enhanced antifungal efficacy of amphotericin B liposomes in tuftsin-loaded liposomes.10 In order to get more insight into the antifungal activity of amphotericin B in vivo, biodistribution of the drug was also analysed in various organs.

Liposomes accumulate in infected tissues, where they release amphotericin B to bind to ergosterol present in fungal cell membrane. Another potential mechanism contributing to decreased nephrotoxicity of liposomal amphotericin B is the preferential diffusion of the amphotericin B molecules to fungal cell membranes versus mammalian cell membranes.21 The release of amphotericin B molecules from the lipid vehicle via fungal phospholipases onto the fungal cell membrane may further enhance the host–pathogen specificity of lipid formulations of amphotericin B.22

From the observations made in the present study, we infer that lipid formulations of amphotericin B possess extra advantages over amphotericin B deoxycholate due to reduced toxicity, more stability and specific targeting of the drug to the site of infection. Further, the liposomal preparation of amphotericin B with immunomodulator tuftsin on the surface not only preserves all the advantages associated with conventional liposomes, but also activates the key components of the immune system (macrophages and neutrophils) for the increased phagocytosis of the pathogens. Therefore, the finding that the efficacy of antifungal agents is increased by addition of tuftsin may be of great clinical significance in cases of immunocompromised persons, the main sufferers of fungal infections.

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Transparency declarations
No declarations were made by the authors of this paper.

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