Toxicity and efficacy differences between liposomal amphotericin B formulations in uninfected and *Aspergillus fumigatus* infected mice

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Abstract

Because of the reduced toxicity associated with liposomal amphotericin B preparations, different amphotericin B liposome products have been made. In the present study, we compared the amphotericin B liposomal formulations, AmBisome® (AmBi) and Lambin® (Lbn), in uninfected and *Aspergillus fumigatus* infected mice, using several *in vitro* and *in vivo* toxicity and efficacy assays. The results showed that the formulations were significantly different, with Lbn 1.6-fold larger than AmBi. Lbn was also more toxic than AmBi based on the RBC potassium release assay and intravenous dosing in uninfected mice given a single 50 mg/kg dose (80% mortality for Lbn vs. 0% for AmBi). Renal tubular changes after intravenous daily dosing for 14 days were seen in uninfected mice given 5 mg/kg Lbn but not with AmBi. Survival following *A. fumigatus* challenge was 30% for 10 mg/kg Lbn and 60% for 10 mg/kg AmBi. When the BAL and lungs were collected 24 h after the second treatment, AmBi at 10 or 15 mg/kg or 15 mg/kg Lbn lowered the BAL fungal burden significantly vs. the controls (*P* ≤ 0.05), while there was no difference in lung fungal burden amongst the groups. In contrast, lung histopathology at this same early timepoint showed that AmBi was associated with markedly fewer fungal elements and less lung tissue damage than Lbn. In conclusion, given the differences in size, toxicity, and efficacy, AmBi and Lbn were not physically or functionally comparable, and these differences underscore the need for adequate testing when comparing amphotericin B liposome formulations.

Key words: aspergillosis, intravenous toxicity, liposomal amphotericin B, nephrotoxicity, RBC toxicity.

Introduction

Liposomes as drug carriers have a long history of being able to reduce the toxic side effects of anti-cancer drugs such as doxorubicin [1], daunorubicin [2,3], cytarabine [4,5], and vincristine [6]. Liposomes and other lipid formulations have also been used to reduce the toxicity of the antifungal drug amphotericin B. These amphotericin B lipid
formulations vary widely in their composition. The commercial lipid formulations of amphotericin B used most extensively include Amphotec®, Abelcet®, and AmBisome® (AmBi) [7,8]. Amphotec is a colloidal disc-like complex of amphotericin B and cholesterol sulfate [9]. Abelcet is a ribbon-like complex of amphotericin B, dimyristoyl phosphatidylcholine (DMPC), and dimyristoyl phosphatidylglycerol (DMPG) [10]. AmBi is a unilamellar liposome composed of amphotericin B, hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and distearoyl phosphatidylglycerol (DSPG) [11]. Fungisome™, which is another liposomal amphotericin B product is composed of amphotericin B, soy phosphatidylcholine, and cholesterol and is marketed in India. The manufacturing process for Fungisome results in multilamellar vesicles that require ultrasonication for 45 min prior to their infusion in order to produce the small unilamellar vesicles suitable for therapy [12].

Given the sensitivity of the manufacturing conditions for these amphotericin B drug carriers, even formulations made up of the same lipid components but produced using different methods, can vary in their physical, pharmacokinetic and pharmacodynamic properties [13]. For example, a liposome formulation of amphotericin B (Anfogen®) previously licensed in Argentina, was reported to have the same chemical composition as AmBisome® but was manufactured differently and subsequently found to have very different physical properties and was much more toxic than AmBisome [14].

There have been several published studies on the animal and human pharmacokinetics of some of these amphotericin B lipid formulations including AmBisome [15–23], Abelcet [24,25], and Amphotec [26]. What is clear from these previous amphotericin B lipid publications is that the presence of infection, the fungal species causing the infection, the extent of spread of the infection (localized or disseminated), as well as the immune status of the host can play a role in determining dose tolerance [20,27–30]. For example, inflammatory cytokines were upregulated in a disseminated Aspergillus flavus murine model treated with 10 mg/kg AmBi, but no upregulation of the inflammatory cytokines was detected in uninfected mice similarly treated [30]. In another study comparing infected and uninfected mice, 20 mg/kg Abelcet in uninfected mice was associated with minimal ongoing renal tubular damage and evidence of tubular repair (regeneration), while in Aspergillus infected mice given the same amount of Abelcet, renal tubular damage was more severe, characterized by acute tubular necrosis and accompanied by surrounding interstitial hemorrhage. Treatment with 20 mg/kg AmBi in uninfected or Aspergillus infected animals showed normal renal tubule morphology [20].

In the present study, we compared two liposomal amphotericin B formulations, AmBi and Lambin® (Lbn), having similar chemical compositions but manufactured using different processes. We evaluated their physical properties as well as their toxicity in uninfected mice and their toxicity, drug distribution, and efficacy in a murine pulmonary Aspergillus fumigatus model.

Materials and methods

Mice

Female C57Bl/6 mice, 6 weeks old at the start of treatment (Harlan International, Indianapolis, IN), were used in the toxicity studies of uninfected animals. We selected this inbred mouse strain because it is highly sensitive to the toxic effects of amphotericin B [31]. Female Swiss Webster mice, 7 weeks old at the start of the treatment (Harlan International, Indianapolis, IN), were used for the studies with Aspergillus fumigatus infected animals since this is an outbred strain of mice with a heterogeneous genetic background somewhat more representative of the genetic diversity in the human population. Animals were maintained in microisolator cages on a standard rodent diet (Teklad Laboratory rodent diet no. 2918 [18% protein]; Harlan/Teklad, Madison, WI) with water ad libitum. All animal research procedures were approved by the Institutional Animal Care and Use Committee of California State Polytechnic University, Pomona.

Test substances

Lyophilized AmBi (AmBisome, Gilead Sciences, Inc. San Dimas, CA) and lyophilized Lbn (Lambin, Sun Pharmaceuticals Ind. Ltd., Halol, India) were reconstituted according to the manufacturers’ instructions to provide final concentrations of 4 mg/ml of amphotericin B for each preparation. Lbn was analyzed by HPLC at Gilead Sciences Inc. and the content of hydrogenated soy phosphatidylcholine, distearoyl phosphatidyl glycerol, cholesterol, and amphotericin B was consistent with the composition of AmBi (data not shown). Both AmBi and Lbn were diluted in sterile 5% dextrose (D5W) for intravenous (i.v.) injection. For the in vitro assay of potassium release from red blood cells (RBCs), the cells were purchased from Bioreclamation, Inc. (Hicksville, NY).

Particle size determination

Aliquots from each of four vials from one lot of AmBi and two vials from one lot of Lbn were analyzed for volume-weighted median particle size and size distribution...
parameters as determined by controlled reference dynamic light scattering using a Micrortrac ultrafine particle analyzer (Honeywell, Morris Township, NJ) [32].

In vitro RBC potassium release toxicity assay

The method of Jensen et al. [33] was used for the assay of potassium release from RBCs. A serial dilution of each drug was prepared with D5W as the diluent to provide a range of amphotericin B concentrations from 0.006% (0.24 μg/ml of AmBi or Lbn) to 12.5% (500 μg/ml AmBi or Lbn) of the original suspension. For each dilution, 50 μl was mixed with 450 μl of washed rat RBCs (Bioreclamation Inc., Hickville, NY), and the mixtures were incubated at 37°C for 12 h. After incubation, the supernatant from each assay dilution was collected, diluted four-fold with 0.9% (w/v) sodium chloride, and measured by a potassium selective electrode in a Nova-1 clinical analyzer (Nova Biomedical, Waltham, MA). The baseline level (0% release) was then defined as the amount of potassium released from the RBCs after incubation with buffer (147 mM NaCl, 3 mM KCl, 10 mM dibasic sodium phosphate, pH 7.4) (negative control); 100% release was defined as the amount of potassium released from the RBCs after incubation with 10 μM valinomycin (CalBiochem, La Jolla, CA) (positive control). For each agent, the concentration of amphotericin B that produced 50% release of the potassium from the RBCs (K₅₀ value) was then calculated.

Single- and multiple-dose in vivo toxicity studies

Uninfected (nonimmunosuppressed) C57Bl/6 female mice, 6 weeks old (n = 5 per group), were given a single i.v. dose of 20 or 50 mg/kg of AmBi or Lbn in a volume of 0.07–0.22 ml. The mice were monitored daily over a period of 14 days for survival, weight gain or loss, grooming, and activity level (general ambulation, laying down, getting up, and breathing difficulty). If an animal died prior to day 14, the weight and disease signs of the mouse on the day of death was included in the average for that group for the remainder of the study. In another study, uninfected C57Bl/6 female mice, 6 weeks old (n = 7 per group), were treated i.v. every day for 14 days with 5.0, 15, or 25 mg/kg of AmBi or Lbn. The mice were again monitored as described above. Blood was collected by cardiac puncture from the uninfected, multidose treated mice 24 h after the last dose of the drug, and the sera were analyzed for blood urea nitrogen (BUN) levels. Histopathological evaluation was also done on mouse kidneys collected at the same time point. At necropsy, kidneys were fixed in 10% neutral buffered formalin. Fixed tissues were processed routinely and stained with hematoxylin and eosin (H&E) for evaluation by a licensed Board-certified veterinary pathologist (Diplomat of the American College of Veterinary Pathologists), (Charles River Laboratories, Davis, CA). Kidney tissues were examined for evidence of treatment-related changes, and severity scores were assigned as follows: minimal (fewer than 25% of tubules affected), mild (25% to 50% of tubules affected), moderate (50% to 75% of tubules affected), and severe (more than 75% of tubules affected).

Efficacy and toxicity testing with Aspergillus-infected mice

Swiss Webster female mice, 7 weeks old, were immunosuppressed intraperitoneally with triamcinolone acetonide at 6 mg/kg (Kenalog-10; Bristol-Myers Squibb Co., Princeton, NJ) on day –3, day 0, and day +2 relative to challenge. The mice were sedated on day 0 with an intraperitoneal injection of 16 mg/kg of xylazine and 80 mg/kg of ketamine and challenged intranasally with 5.9 × 10⁶ A. fumigatus conidia as previously described [34]. Drug treatment was initiated 2 h post-challenge, with five groups of mice (n = 22 per group) receiving 10 or 15 mg/kg of i.v. AmBi or Lbn in a volume of 0.08–0.11 ml. The control group of mice was given i.v. D5W. Ten mice in each group were monitored for survival, weight gain or loss, and disease signs for 21 days, with additional treatments every 24 h for a total of six treatments post-challenge. The clinical signs of infection were scored daily based on weight loss, the level of grooming, and activity level (general ambulation, laying down, getting up, and breathing difficulty). If an animal died prior to day 21, the weight and disease signs of the mouse on the day of death was included in the average for that group for the rest of the study. The remaining twelve mice in each group were given a total of only two treatments, one at 2 h and another at 24 h post-challenge and were killed at 48 h post-challenge for fungal burden determination, tissue drug concentration, and serum BUN analysis (n = 7 per group); histopathology was done on the tissues of the other five mice per group. For fungal burden determination, the lungs were collected aseptically, weighed, homogenized in 1 ml of phosphate-buffered saline (PBS), diluted in PBS, and 200 μl aliquots of each dilution were plated in duplicate onto Sabouraud agar plates and the plates incubated at 30°C for 24 h to determine the number of cfu per gram of lung tissue [20]. Tissue drug concentration was determined as previously described [34]. Briefly, a 200 μl aliquot of each lung homogenate was mixed with 200 μl methanol, heated in a 65°C water bath for 10 min, centrifuged at 1000 × g for 8 min, the supernatant collected, and analyzed in a bioassay using Candida albicans (limit of sensitivity = 0.125 μg/ml). Blood from these same mice was taken by cardiac puncture, and their sera were analyzed...
Table 1. Median particle diameters and 90% passing diameters as determined by dynamic light scattering for individual vials of AmBi and Lbn.

<table>
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<th>Vial</th>
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<th>Diameter (nm) of particles in Lbn</th>
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<tr>
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<td>Median 90% Passing</td>
<td>Median 90% Passing</td>
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<tr>
<td>1</td>
<td>75.9 115.3</td>
<td>121.0 174.9</td>
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<td>2</td>
<td>76.9 126.5</td>
<td>123.3 165.6</td>
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<td>4</td>
<td>77.5 123.2</td>
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<td>Avg (SD)</td>
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for BUN levels. For histopathological analysis, kidneys and lungs were collected and processed for histopathology as described above. Infected kidney tissues were examined for evidence of treatment-related changes and severity scores of minimal, mild, moderate, and severe were assigned as described above. Scoring of the infected lungs reflected both the severity and the distribution of changes. The lungs were assessed for the presence of mixed neutrophilic, histiocytic alveolar infiltrate (alveolar exudates), neutrophilic exudates in large airways, tissue necrosis, alveolar hemorrhage and edema, and vascular invasion.

Statistical analysis

Tissue drug concentration (micrograms per gram) and tissue fungal burdens (cfu per gram) were analyzed by using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA). A Kruskal–Wallis nonparametric analysis of variance was applied to compare the control to all groups in each experiment, and where differences occurred, a two-tailed Mann–Whitney U test was used for paired-group comparisons. Survival curves were compared using the log rank test. A P-value of ≤0.05 was considered significant.

Results

Particle size determination

The reconstituted materials in each vial were examined for median particle size and for the upper limit particle diameter of 90% of the particles. The latter measure is referred to as the 90% passing diameter and is an indicator of the presence of particles larger than 100 nm in a small, unilamellar vesicle dispersion [32]. For AmBi, the median particle size for the four vials examined was 77.8 +/− 2.2 nm (SD) within the validated method precision of 3.5% [32]. For Lbn, two vials were examined and the median particle size was 122.2 +/− 1.6 nm, which was 1.6 fold larger than the particle size for AmBi. The 90% passing diameter values for all four AmBi vials averaged 122.0 +/− 4.8 nm, but the 90% passing diameter of Lbn was 170.3 +/− 6.6 nm, which was 28.4% larger than AmBi, reflective of a substantially shifted size distribution for Lbn indicating the presence of substantial amounts of particles larger than 100 nm (Table 1).

**In vitro RBC potassium release toxicity assay**

The propensity for amphotericin B in each formulation to partition into the cell membrane of RBCs during incubation can be used as one measure of toxicity by determining the concentration of amphotericin B required to achieve 50% potassium release (K$_{50}$) from washed rat RBCs after 12 h of incubation [33]. The concentrations leading to 50% potassium release (K$_{50}$) in this study were 1.4 μg/ml for Lbn and 7.4 μg/ml for AmBi (Fig. 1). No vial-to-vial variability was noted for either product in terms of the resulting K$_{50}$. Fungizone (deoxycholate amphotericin B) is normally evaluated in this assay after 4 h of incubation and would be off scale (fully partitioned) with a 12-h incubation time. The K$_{50}$ results indicate a substantially greater propensity for amphotericin B to partition out of Lbn relative to AmBi, and a prediction of enhanced toxicity on infusion and/or at the site of accumulation of the liposomes in vivo.

Figure 1. Potassium (K50) Release from rat red blood cells (RBCs) measured after 12 h of incubation at 37°C with dilutions of Lbn or AmBi. K50 values: Lbn (1.4 μg/ml); AmBi (7.3 μg/ml).
Figure 2. Single-dose i.v. toxicity of Lbn or AmBi in uninfected female C57Bl/6 mice (n = 5/group). (A) Survival, 50 mg/kg Lbn vs. 20 or 50 mg/kg AmBi or 20 mg/kg Lbn (P < 0.001). (B) Mean percent weight change, 50 mg/kg Lbn vs. 20 or 50 mg/kg AmBi or 20 mg/kg Lbn (P < 0.001).

Uninfected mice: single-dose in vivo toxicity studies

To determine if the increased toxicity of Lbn observed in the in vitro K50 RBC assay was also seen in vivo, we tested the formulations in vivo by intravenously administering single increasing doses of AmBi or Lbn. There were no deaths in the mice given a single dose of 20 mg/kg of AmBi or Lbn with no weight loss during the 14 days of monitoring (Fig. 2A, B). However, a single dose of 50 mg/kg Lbn produced severe weight loss and 80% mortality. In comparison, all the mice given a single dose of 50 mg/kg AmBi survived causing only a slight drop in weight the first day after injection. This in vivo toxicity study confirmed the toxicity differences in the formulations predicted by the in vitro RBC assay (Fig. 1).

Uninfected mice: multiple-dose in vivo toxicity studies

Since the recommended clinical treatment for liposomal amphotericin B is dosing for a period of 2 to 4 weeks, depending upon the patient and the type of fungal infection [17,35,36], we compared the toxicities of two liposome products in a multiple-dose setting in uninfected mice. Doses of each drug were selected based on previous experience with AmBi [14] and the in vivo toxicity single dose study reported above. The multiple-dose testing included 14 daily i.v. injections of 5.0, 15, or 25 mg/kg AmBi or Lbn. None of the animals died at these doses, and all test groups showed a consistent weight gain throughout the 14-day study, similar to that of the control D5W mice (data not shown). However, toxicity differences were observed between the formulations at the tissue level when examined histologically. Renal tubular changes were not identified in any of the D5W control group kidneys (Fig. 3A). In comparison, tubular changes with Lbn treatment, noted as minimal or mild degeneration/regeneration, were identified at 5.0 mg/kg Lbn (Fig. 3C), while treatment with 5.0 mg/kg AmBi was not associated with renal tubular changes in any of the mice in this AmBi dose group (Fig. 3B). Renal tubular changes were increased in incidence in a dose dependent manner between the 5.0 and 15 mg/kg Lbn groups, and there was no appreciable difference in the incidence or severity of renal tubular changes between the 15 and 25 mg/kg Lbn groups. Tubular changes in the 15 and 25 mg/kg AmBi treatment groups were predominantly minimal in severity. Only one 25 mg/kg AmBi dose group mouse had mild tubular changes.

A. fumigatus infected mice: survival and disease signs

To examine the effect of daily dosing for six days in infected animals, we tested these drugs in a murine model of pulmonary A. fumigatus infection. Mice in the control D5W group succumbed readily to the infection. All deaths occurred between 24 h and 72 h post-challenge (Fig. 4A) with a survival rate of 10% by the end of the study and maximum weight loss on day 6 of 18% (Fig. 4B) (survival, P ≤ 0.02 for D5W vs. 10 or 15 mg/kg AmBi or 15 mg/kg Lamb). The number of deaths in the Lbn and AmBi treatment groups given four doses was comparable up to day 3, but even with two additional drug doses for the surviving mice, animals in the 10 mg/kg Lbn group continued to die, with 30% survival by the end of the study. The mice in the 10 mg/kg Lbn group had a greater percent weight loss than the control D5W mice, and disease sign scores that were more severe than the other drug treatment groups (Fig. 4B, C). In comparison, survival of mice treated with 10 or 15 mg/kg AmBi was 60% and 50%, respectively, with the least overall weight loss and mild disease signs for the animals in the 10 mg/kg AmBi group. In the 15 mg/kg Lbn treated mice, survival was 60%, with weight loss and disease sign scores similar to that observed with 10 mg/kg AmBi.
**Figure 3.** Kidney histopathology (n = 7/group) in uninfected female C57Bl/6 mice. Representative mice from each group are depicted. (A) Normal renal tubules in hematoxylin and eosin (HE)-stained kidney from mouse treated i.v. daily for 14 days with 5% dextrose (D5W, control). Bar, 100 μm. (B) HE-stained kidney from mouse treated i.v. for 14 days with 5 mg/kg AmBi, with normal renal tubular morphology similar to D5W mice. (C) Renal tubular changes seen in all mice, except one, treated i.v. for 14 days with 5 mg/kg Lbn with tubular dilation, protein casts (arrows) and/or tubular regeneration ranging from minimal to mild.

**A. fumigatus** infected mice: fungal burden in the BAL and lungs

To obtain more information about the efficacy of these two drugs, we examined how effective these drugs were in reducing the fungal burden. The *Aspergillus* infected mice were evaluated for cfu/ml in the BAL and cfu/g in the lungs at 48 h post-challenge when the animals had only received two drug treatments (Fig. 5). We had to collect the tissues at this early timepoint because of the severity of the infection. We could not wait until after six treatments, as was done in the mice that were followed for survival. Although Lbn at 10 mg/kg had a significantly higher fungal burden in the BAL compared to 10 or 15 mg/kg AmBi (P ≤ 0.05)
Figure 5. Fungal burden (cfu/g lung and cfu/ml BAL) of female Swiss Webster mice (n = 7/group), suppressed with triamcinolone, and challenged intranasally with A. fumigatus (5.6 × 10^6 conidia/mouse) 24 h after the second i.v. treatment with 5% dextrose (D5W), or 10 or 15 mg/kg Lbn or AmBi. (A) In BAL, 10 mg/kg Lbn vs. 10 or 15 mg/kg AmBi and 15 mg/kg Lbn (P ≤ 0.05). (B) In lungs, D5W vs. 10 or 15 mg/kg Lbn and 15 mg/kg AmBi (P ≤ 0.01). Bar = mean/group.

(Fig. 5A), the fungal burden in the BAL with all the liposome treatments was the same as that seen in the D5W control group. In the lungs, two treatments with either 10 or 15 mg/kg Lbn or 15 mg/kg AmBi produced significantly lower fungal burdens compared to the D5W control (P ≤ 0.01), but there were no significant differences in reduction of lung fungal burden amongst any of the drug treatment groups (P > 0.05) (Fig. 5B). A better correlation between survival and reduction of lung fungal burden would probably have been observed if the lung samples had been collected after six treatments instead of only two treatments.

**A. fumigatus** infected mice: drug concentrations in the BAL and lungs

Because we observed that the 10 mg/kg AmBi produced more prolonged survival at day 21 compared to Lbn at 10 mg/kg, we investigated whether this difference was related to the drug concentration in these tissues collected at an early timepoint. In the lung tissue, there was no statistically significant difference in drug concentration between Lbn and AmBi at either dose (Fig. 6A). In the BAL, there was also no significant difference in drug concentration between the liposome formulations (Fig. 6B). However, with 15 mg/kg dosing, six out of seven animals had no detectable drug in the BAL following 15 mg/kg Lbn treatment, while five out of seven animals given 15 mg/kg AmBi had drug levels that ranged from 2.6 to 8.0 μg/ml. Despite these results, the difference in BAL concentration between the two drugs at this dose did not reach significance (P = 0.06). These results indicated that prolonged survival could not be correlated with the lung or BAL drug concentrations when the analysis was done following only two drug treatments.
**A. fumigatus** infected mice: lung histopathological evaluation of toxicity and efficacy

We also examined the lung tissues after two drug treatments to evaluate tissue toxicity and fungal penetration. Hematoxylin and eosin stained lung sections were evaluated for efficacy and drug toxicity, with Gridley stained sections aiding in the identification of fungi. In the lung tissue, there was severe bronchopneumonia in all mice (Fig. 7). In the control D5W treated mice, abundant intralelional conidia and hyphae were associated with acute, severe, necrotizing bronchoalveolar inflammation, vasculitis, vascular congestion and pulmonary edema (Fig. 7A, B). In the 10 mg/kg Lbn-treated mice, there was ongoing, bronchiolar necrosis in some areas, as well as bronchiolar epithelial hyperplasia in others, indicative of a regenerative response (Fig. 7C), which was not present in the controls. There was also a lot of intralelesional fungi, but they were less abundant than that found in the controls, with more conidial than hyphal forms (Fig. 7D). In the 10 mg/kg AmBi group, lungs displayed a similar degree of pulmonary inflammation as that of mice treated with 10 mg/kg Lbn or D5W, but the extent of pulmonary edema and hemorrhage was less (Fig. 7E). Some conidia were seen, but the occurrence of hyphae was rare in the 10 mg/kg AmBi group (Fig. 7F). Infected mice treated with 15 mg/kg Lbn presented with inflammation comparable to that found in mice treated with 10 mg/kg Lbn with hyperplastic epithelium lining the bronchiole but without evidence of vascular invasion (Fig. 7G) and the presence of some fungal elements (Fig. 7H). Although the severity of the pulmonary inflammation seen in infected mice treated with 15 mg/kg AmBi was comparable to that seen in the other groups, there was no evidence of microabscess formation or vascular necrosis and hyphal forms were less prevalent (Fig. 7I, J). Overall, AmBi was associated with less lung tissue damage and reduced fungal penetration compared to Lbn when the tissues were examined microscopically. This difference in lung efficacy indicates that in this model at an early timepoint, long-term survival correlated better with the lung histopathologic observations as compared to evaluation of the lung fungal burden using cfu/g tissue analysis.

**A. fumigatus** infected mice: kidney histopathology and BUN evaluation of toxicity

As renal tubular changes had been noted in uninfected mice given 5 mg/kg Lbn every day for 14 days, the blood and kidneys of infected mice were collected at 24 h after only two drug doses to determine whether any toxicity was evident in the infected kidneys at this early timepoint. As mentioned above, we had to collect the tissues at 48 h post-challenge due to several deaths that occurred in some of the groups, particularly the D5W group. The kidneys of D5W treated mice had normal tubular structure. However, mice given either Lbn or AmBi had some evidence of minimal renal changes, which increased in a dose dependent manner for both drugs. There was tubular dilation, protein casts, and single cell necrosis, and these effects were slightly more evident in the AmBi treatment groups. After only two drug treatments, BUN levels in all mice were within normal limits (12 to 39 mg/dl serum) [37].

**Discussion**

The toxic effects of the antifungal drug, deoxycholate amphotericin B, have been markedly reduced by its formulation into liposomes. However, as reported previously in a study of Anfogen and AmBi, if the amphotericin B liposomes are not manufactured under the same conditions, even though the liposomes have the same chemical composition, their physical properties as well as toxicity and efficacy profiles can be very different [14]. In the present study, the liposomal amphotericin B products, Lbn and AmBi, also seem to have the same chemical components but they have different particle sizes, with Lbn being 1.6-fold larger than AmBi (122.2 nm Lbn vs. 77.8 nm AmBi). This difference in size can affect how the liposomes distribute in the host and their rate of cellular uptake [38].

The conventional in vitro toxicity assay for amphotericin B is the RBC lytic assay [39]. RBCs are used because they have high levels of cholesterol in their membranes to which the amphotericin B will bind resulting in pore formation and lysis of the cells. A more sensitive RBC assay measures the leakage of potassium from the RBCs when they are initially damaged by the amphotericin B and has been used successfully to compare the in vitro toxicity of different amphotericin B lipid formulations [33]. When the potassium release assay was used in this study, AmBi required 5× more drug than Lbn to produce 50% potassium leakage of the RBCs (K50 = 1.4 μg/ml Lbn vs. 7.4 μg/ml AmBi) demonstrating the reduced in vitro toxicity of AmBi relative to Lbn.

In vitro assays are helpful in identifying toxicity at the cellular level, and in the case of lipid amphotericin B formulations, indicative of the binding affinity of the amphotericin B for the liposome bilayer vs. the mammalian cell membrane. However, in vivo toxicity testing is needed to evaluate how the pharmacodynamics of the drug will affect its toxicity. In the present study, we evaluated both liposomal amphotericin B drugs in uninfected C57Bl/6 mice following either a single injection or daily treatment for 14 days. We used this extended therapy since amphotericin B treatment of fungal infections requires repeated dosing.
Figure 7. Lung histopathology (HE or Gridley stain) of Swiss Webster mice, suppressed with triamcinolone and challenged with A. fumigatus ($5.6 \times 10^6$ conidia/mouse), with lung collection 24 h after the second i.v. treatment with 5% dextrose (D5W), or 10 or 15 mg/kg Lbn or AmBi. Representative mice from each group are depicted ($n = 5$ group). (D5W group: A, B) Inflammation and abundant intralesional conidia and hyphae, necrosis, vascular congestion, and edema. (10 mg/kg Lbn group: C, D) Severe bronchopneumonia, with inflammation, pulmonary edema, and/or hemorrhage similar to that in the control D5W group, with large amounts of conidia and hyphae in all mice treated. (10 mg/kg AmBi group: E, F) Inflammation comparable to that with 10 mg/kg Lbn or D5W but less pulmonary edema and hemorrhage, along with some conidia and rare fragmented hyphae. (15 mg/kg Lbn group: G, H) Inflammation similar to that in mice given 10 mg/kg Lbn but with reduced amounts of conidia and hyphae. (15 mg/kg AmBi group: I, J) Similar inflammation to that with 10 mg/kg AmBi but with mild epithelial necrosis, no microabscess formation, and few hyphal elements. B = bronchial epithelium, BV = blood vessel, TA = terminal airway, C = conidia, H = hyphae. Bar for HE-stained lungs (A, C, E, G, I) = 1 mm. Bar for Gridley-stained lungs (B, D, F, H, J) = 50 μm.
over a period of several weeks [40–42]. Although all the uninfected mice given a single i.v. injection of 20 mg/kg Lbn or AmBi survived, all but one mouse given a single dose of 50 mg/kg Lbn died, while there were no deaths in those mice given a single dose of 50 mg/kg AmBi. When uninfected animals received multiple drug treatments (5, 15, or 25 mg/kg/day) for 14 days, evidence of chronic kidney damage was detected microscopically in those mice given 5 mg/kg Lbn but not in the kidneys of mice treated with 5 mg/kg AmBi. This difference is significant since the clinical dose of AmBi is 3–5 mg/kg in patients being treated for fungal infections and treatment is often for at least 2 to 4 weeks [17,35,36,43–45]. Furthermore, amphotericin B treatment in an already immunosuppressed patient has to be considered carefully, especially if they are being given other nephrotoxic or hepatotoxic drugs [46–48].

Although there were clear toxicity differences between the two formulations based on our in vitro assays and in vivo intravenous toxicity testing in uninfected animals, animals infected with A. fumigatus do not always tolerate repeated dosing with the same high doses of amphotericin B lipid formulations as the uninfected animals [14,20]. To address this issue, in the present study we used the steroid immunosuppressed pulmonary A. fumigatus murine model for evaluating the efficacy of daily intravenous treatment with AmBi or Lbn in infected mice. We administered 10 or 15 mg/kg of each drug since those doses have been reported to be most effective in previous mouse aspergillosis studies [14,25,43]. AmBi at either dose (10 or 15 mg/kg) given every day for 6 days significantly prolonged survival, whereas only the higher dose of Lbn (15 mg/kg) increased survival. Thus, administering Lbn at the required higher dose could potentially result in increased toxicity since antifungal treatment often requires 2–4 weeks of therapy.

It has been reported that if Aspergillus infected tissue is collected after at least four drug treatments, there is a correlation between reduction in fungal burden and drug concentration in the tissues [20]. However, due to the severity of the infection in the present study, we had to collect tissues after only 2 days of treatment to obtain enough tissue samples for each group. We observed significantly lower cfu/g lung in the 10 or 15 mg/kg Lbn and 15 mg/g AmBi groups compared to the D5W control mice, while there was no significant difference in efficacy amongst any of the drug treatment groups. Similarly, the amount of drug in the lungs was the same for all drug treatments. This would suggest a possible correlation between reduction in cfu/g lung and lung drug concentration. However, histopathologic evaluation of the lung tissues even at this early timepoint showed that AmBi treatment at either dose was more effective than Lbn with less lung tissue damage and fewer fungal elements, particularly at the 10 mg/kg dose. This correlated better with the prolonged survival achieved with 10 mg/kg AmBi vs. 10 mg/kg Lbn. As reported in other studies comparing amphotericin B formulations in pulmonary and central nervous system aspergillosis, important additional information about drug efficacy and toxicity was obtained through histologic examination of infected tissues, which complemented traditional methods of fungal burden assessment and toxicity testing [49,50]. These studies underscore the need to include histological examination of target tissues when evaluating drug efficacy and toxicity of lipid amphotericin B formulations. Furthermore, in subsequent studies, consideration should be given to using an inhalation mouse pulmonary aspergillosis challenge model as it would be less acute and would allow a longer-term follow up with multiple sampling times to evaluate toxicity [51].

The observations from this study re-enforce the conclusion that the process used to formulate a drug is critical with respect to ensuring that it has the desired physical and pharmacokinetic/pharmacodynamic properties. This is particularly important when the drug has to be incorporated into a carrier system, such as a liposome or other nanoparticle. Consequently, it is essential that supposedly comparable antifungal drugs prepared under different processing conditions be tested in both uninfected and fungal infected animals, using a range of in vitro and in vivo toxicity and efficacy assays. By using this multiple testing approach, we have shown that there were significant differences between Lbn and AmBi based on their physical properties as well as their biological activity in both uninfected and Aspergillus infected mice. This type of analysis for evaluating similarities and differences among antifungal drugs can be readily applied to other antimicrobial agents and is of particular importance when examining drugs incorporated into carrier systems.

**Declaration of Interest**

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