Effects of dosing regimen on accumulation, retention and prophylactic efficacy of liposomal amphotericin B

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Objectives: We hypothesized that effective prophylactic treatment of fungal infections would require adequate drug penetration and retention at potential infection sites. Using a mouse model, we examined liposomal amphotericin B (L-AmB) biodistribution, cell localization and retention in kidneys, lungs, liver and spleen to evaluate effective dosing regimens for prophylaxis of Candida glabrata and Candida albicans infections.

Methods: Following treatment of mice with cumulative doses of L-AmB (60–225 mg/kg), a bioassay was done to determine tissue drug concentrations 12 h to 6 weeks post-treatment. Immunohistochemical staining with anti-amphotericin B antibodies was used for cellular drug localization. Mice were treated prophylactically with 15–90 mg/kg L-AmB and challenged intravenously 1–7 days later with C. glabrata or they were given a total of 60 mg/kg as daily or intermittent dosing followed by intravenous challenge with C. albicans or they were given a total of 60 mg/kg as daily or intermittent dosing followed by intravenous challenge with C. albicans 3 or 6 weeks later.

Results: On the basis of μg/g tissue, the relative amount of drug was in the order spleen > liver > kidneys > lungs. Amphotericin B levels were maintained above the MIC for many fungi for 1 week in lungs and for as long as 6 weeks in kidneys and spleen. Drug localized in kidney tubular epithelial cells and in macrophages of liver and spleen. In prophylactic models, fungal burden was reduced by several 1000-fold or was undetectable within target tissues (kidneys, spleen).

Conclusions: These observations underscore the importance of including drug tissue levels to obtain a better understanding of L-AmB efficacy. The sustained concentrations of bioactive AmB in many tissues provide a further rationale for investigating L-AmB prophylactic regimens.

Keywords: lipid formulations, bioavailability, candidiasis, antifungal agents

Introduction

Effective treatment of fungal infections requires adequate drug penetration and retention at the sites of infection. To achieve this, pharmacokinetic studies are traditionally done to measure peak drug levels and AUC in the blood to help develop dosing regimens that will maintain the drug in the blood for several hours at, or preferably above, the MIC for the infecting agent.1,2 Although this approach is helpful, it is not adequate to address the issue of drug accumulation and retention within tissues such as the kidneys, lungs, liver and spleen, which can be the sites of fungal growth during an infection. Since diagnosis of fungal infections is often delayed,3 the fungal burden within the tissues can be considerable at the time of initiation of treatment, requiring more drug to be delivered to the tissues to effectively reduce the fungal burden.

When a given drug is delivered within a carrier system, such as a liposome or a lipid complex, the tissue distribution of the drug can be modified. This is exemplified by comparing the pharmacokinetics and tissue distribution of amphotericin B deoxycholate and the different lipid formulations of amphotericin B, such as amphotericin B lipid complex (ABLC; Abelcet®) and liposomal amphotericin B (L-AmB; AmBisome®). For L-AmB, the peak plasma amphotericin B

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concentration was at least 25 times greater, and the AUC was at least 15 times greater, than that of other lipid formulations of amphotericin B and amphotericin B deoxycholate in experimental animals. 6,9 – 11 Olson et al. 6 reported that in mice given an intranasal challenge with Aspergillus fumigatus, multidose treatments at 15 mg/kg with ABLC resulted in higher drug levels in the lungs and the spleen than treatments with L-AmB, whereas L-AmB treatments gave higher drug levels in the kidneys and the blood. Drug levels in the liver were comparable for both treatments. Even though the two lipid amphotericin B formulations differed in the amount of drug that they delivered to the lungs, there was no significant difference in fungal burden between ABLC and L-AmB at 15 mg/kg, although ABLC showed a significantly greater reduction at 20 mg/kg compared with L-AmB at 20 mg/kg.

The lipid formulations of amphotericin B were developed to reduce the dose-limiting toxicities of amphotericin B, 6 and clinical studies have demonstrated that L-AmB is the least toxic of the commercially available formulations of amphotericin B. 7,8 L-AmB has been used for over 10 years for empirical and salvage therapy, 6,9 – 11 as well as for primary treatment of candidiasis or spleens for histoplasmosis. 13 In a recent clinical trial, L-AmB has been shown to be efficacious for the treatment of primary aspergillosis. 14 There have also been some studies on its potential prophylactic use in high-risk patients. 15 – 17

Obtaining a better understanding of the biodistribution of L-AmB is particularly important when considering prophylactic therapy. The correlation between drug concentration and efficacy in a prophylactic setting has been investigated in both murine candidiasis and histoplasmosis, using a single dose of L-AmB with a challenge 7 days later. 18 The results showed a correlation between drug concentration in the target tissue (kidneys for candidiasis or spleens for histoplasmosis) and efficacy based on survival and fungal burden. To further evaluate the use of L-AmB to prevent or treat early fungal infections, studies need to be done to examine the drug’s short-term and long-term accumulation and retention in different tissues using various dosing regimens. The limited pre-clinical data on L-AmB tissue biodistribution has been summarized in a review by Adler-Moore and Proffitt. 19 In the present study, we used a mouse model to examine L-AmB biodistribution, cell localization and retention in kidneys, lungs, liver and spleen following different dosing regimens and then tested the prophylactic efficacy of selected regimens in both non-albicans and Candida albicans systemic infections that targeted to the kidneys and spleen. These experiments demonstrated that L-AmB in both the kidneys and spleen was retained at sufficiently high levels to provide prophylactic efficacy against systemic challenge with Candida species at 3 and 6 weeks post-treatment.

Materials and methods

Animals

Female DBA/2 mice, 5–6 weeks old at the start of treatment (Harlan, Indianapolis, IN, USA), and female C57BL/6N mice, 8–11 weeks old at the start of treatment (Harlan), were used in the studies. Animals were maintained in microisolator cages with standard rodent diet (Lab Mouse Diet no. 5015; PMI Nutrition International, Brentwood, MO, USA) and water ad libitum. All animal research procedures were approved by the Institutional Animal Care and Use Committee of California State Polytechnic University, Pomona, USA.

Test substances

Lyophilized L-AmB (AmBisome®; Gilead Sciences, Inc., San Dimas, CA, USA) was reconstituted with 12 mL of sterile water for injection (Abbot Laboratories, North Chicago, IL, USA) to give 4 mg/mL amphotericin B, shaken vigorously for 1 min and filtered through a 0.5 µm filter according to the manufacturer’s instructions.

Treatment regimens for drug biodistribution studies

The initial study in uninfected DBA/2 mice was done to determine drug concentrations in various organs 7 or 14 days post-treatment. Cumulative doses of 90, 135, 180 or 225 mg/kg L-AmB were administered to groups of DBA/2 mice (n = 5 per group) by intermittent intravenous (iv) injections of 15 mg/kg L-AmB/treatment on Mondays, Wednesdays and Fridays (90 mg/kg = 2 weeks of treatment; 135 mg/kg = 3 weeks of treatment; 180 mg/kg = 4 weeks of treatment; 225 mg/kg = 5 weeks of treatment). Animals in each group were sacrificed by carbon dioxide inhalation either 7 or 14 days after the last treatment and the kidneys, lungs, spleen and liver aseptically removed, weighed and frozen for subsequent analysis of amphotericin B concentration by a modified bioassay. 20 In a subsequent study, groups of uninfected DBA/2 mice (n = 5 per group) were given 180 or 225 mg/kg total dose of L-AmB as described above and sacrificed 12, 24 or 48 h post-treatment. The kidneys, lungs, spleen and liver were removed, weighed and frozen for subsequent bioassay of amphotericin B concentration. An additional 18 uninfected DBA/2 mice were treated with a total dose of 225 mg/kg L-AmB as described above and their blood collected at 0.5, 2.0, 4.0, 8.0, 12 and 24 h (n = 3 mice/time point). Following separation of the serum, the serum samples were analysed by bioassay for drug concentration.

Bioassay for determination of drug concentrations

To determine tissue and serum drug levels, we used a modification of the bioassay described by Shadomy et al. 20 Paecilomyces variotii (ATCC 22319) was cultured on PDA medium (Difco Labs, Sparks, MD, USA) at 40°C for 48 h in the dark to stimulate spore production, followed by incubation for 72 h at room temperature. Spores harvested in saline containing 0.01% Tween 80 were filtered through sterile gauze and the suspension sonicated for 1 min in a water bath sonicator to break up clumps. Counts were determined with a haemocytometer and verified by plating 200 µL in duplicate on Sabouraud’s dextrose agar plates with 0.05% chloramphenicol (Hardy Diagnostics, Santa Maria, CA, USA). cfu were determined after 24 h of incubation at 35°C. The spore suspension was adjusted with 0.01 M PBS, pH 7.2, and added to sterile 2.35% molten antibi-otic medium 19 agar (AM19a; Difco Labs) to give final concentrations of 3 × 10⁴ spores/mL, which was poured into Nunc Bioassay plates (Nalge Nunc International Corp., Rochester, NY, USA). After solidification of the seeded medium, wells were made in the agar using a 7-well template pattern (8 mm diameter/well). L-AmB was diluted 1:1 with methanol and heated for 10 min at 65°C to disrupt the liposomes and then diluted further in PBS to obtain final concentrations of 0.03–1.0 µg/mL amphotericin B. Amphotericin B (Spectrum Chemical Manufacturing Corp, New Brunswick, NJ, USA) was dissolved in DMSO (Sigma, St Louis, USA).
MO, USA), diluted 1:1 with methanol and heated for 10 min at 65°C and then diluted further in PBS to obtain the same final concentrations as the L-AmB. Weighed tissue samples were homogenized (Tissue Tearer, 7 mm microtip probe; Biospec Products, Inc., Bartlesville, OK, USA) in 1 mL of PBS, diluted 1:1 with methanol and then heated for 10 min at 65°C. Serum samples were treated similarly without homogenization. Samples were centrifuged at 950 g for 8 min and the supernatants diluted in PBS. Each sample dilution or drug standard dilution (150 μL) was placed in an agar well, incubated for 1 h at room temperature and then incubated for 22 h at 35°C. Each sample and drug standard dilution was tested in duplicate. The zone of inhibition around each well was measured with a Vernier caliper and the zone size used to determine drug concentration in the samples (μg/g) by linear regression analysis. As a control, tissue homogenates from each of the different organs were spiked with known concentrations of L-AmB and processed as described above using the methanol extraction procedure. The recovery of amphotericin B from the tissues was as follows: 91.3% from lungs, 92.7% from kidneys, 89.6% from spleen and 91.6% from liver. We made the assumption that the drug recovered after the methanol extraction represented bioavailable drug (close to 90%) from liver. We made the assumption that the drug recovered after the methanol extraction represented bioavailable drug (close to 90% from all tissues), and, therefore, the drug levels we have reported in the Figures are non-adjusted values. The sensitivity of the assay was 0.03 μg/mL. The intra-day coefficient of variation ranged from 3.4% to 6.3% and the inter-day coefficient of variation ranged from 9% to 10%.

**Immunohistochemical localization of drug in tissues**

Immunohistochemical detection of amphotericin B was performed using affinity-purified rabbit polyclonal antiserum (serum provided by Gilead Sciences, Inc., Foster City, CA, USA). To purify the anti-amphotericin B antibody and decrease any non-specific binding, the carboxyl end of amphotericin B was attached to a solid support matrix and then bound anti-amphotericin B antibody was separated from the rest of the serum by elution. The bound antibody had specific reactivity with amphotericin B in tissues, and the affinity-purified antibody was concentrated to 1 mg/mL, titrated and determined to have optimal reactivity at a 1:20 dilution.

Kidneys, lungs, livers and spleens (one or two tissue sections/organ/animal) were collected 3 or 7 days after 225 mg/kg cumulative dosing with L-AmB (female DBA/2 mice, n = 2 per group). Tissues were fixed in 10% neutral buffered formalin, embedded, frozen, cryosectioned and stained with affinity-purified anti-amphotericin B antibody using an avidin–biotin–peroxidase complex (ABC) immunoperoxidase procedure. Additional tissue sections were embedded in paraffin, sectioned, stained with haematoxylin and eosin (H&E), evaluated microscopically for background staining. The ABC detection method was used with a protein blocking solution composed of 1% BSA (Sigma), 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA), 5 μg/mL sheared salmon sperm DNA (Eppendorf, Westbury, NY, USA) and one drop of normal mouse serum (Sigma) per 2 mL of protein block. These treatments decreased non-specific background staining.

For the staining procedure, cyrosections were incubated in 3% hydrogen peroxidase (Sigma) for 10 min at room temperature. The slides were rinsed twice with PBS + Tween (PBST; 0.15 M NaCl, pH 7.2 + 0.05% Tween 20), blocked with avidin solution for 15 min, rinsed with PBST and subsequently blocked with biotin solution for 15 min and rinsed with PBST (avidin biotin blocking kit; Vector Laboratories). The protein block was as described above. Following the protein block, anti-amphotericin B or rabbit IgG1 isotype control (Dako, Carpinteria, CA, USA) antibodies were applied to the slides (1:20 dilution for rabbit anti-amphotericin B and 1:100 for rabbit IgG1) and incubated for 1 h at room temperature. The slides were rinsed twice with PBST and biotinylated secondary antibody [1:500 goat anti-rabbit IgG (H+L); Jackson Immunoresearch, West Grove, PA, USA] applied for 30 min, followed by rinsing twice with PBST. The slides were reacted for 30 min with ABC Elite reagent (Vector Laboratories), rinsed twice with PBST and Nova Red (Vector Laboratories) applied for 10 min as a substrate for the peroxidase reaction. All slides were then rinsed with tap water, counterstained with Haematoxylin Blue (Richard-Allan Scientific, Kalamazoo, MI, USA) in saturated lithium carbonate (Sigma), dehydrated through alcohols, cleared in xylene (EMD, Gibbstown, NJ, USA) and coverslipped for interpretation. Amphotericin B deoxycholate (X-Gen Pharmaceuticals, Northport, NY, USA) prepared as a 20 μg/mL solution in water and parathyroid hormone-related protein (non-specific background control) (Sigma) at the same concentration were spotted onto UV-activated resin slides (Instrumeds, Inc., St Louis, MO, USA). UV-adhered to the slide prior to fixation, air-dried, immunostained as described for the cyrosections and used as positive and negative controls, respectively. Anti-amphotericin B staining was compared with the isotype serum control staining in L-AmB-treated animals and placebo-treated controls. Tissue sections were evaluated qualitatively for distribution, frequency and intensity of staining. Only staining above background levels of the isotype serum controls was considered amphotericin-B-specific.

**Fungal inocula**

Beginning 3 days prior to challenge, *C. albicans* (CP strain no. 620) or *Candida glabrata* (ATCC strain 90030) was subcultured daily in Sabouraud’s dextrose broth. On the day of challenge, the subculture was pelleted, rinsed twice with PBS, centrifuged at 1000 g and the final pellet resuspended in PBS. The concentration of blastospores was counted in a haemocytometer and the blastospore suspension adjusted with PBS to produce 1 × 10⁶ blastospores/mL. *C. albicans* or 1 × 10⁷ blastospores/mL *C. glabrata*. Counts were verified by plating 200 μL of a diluted blastospore suspension in duplicate on Sabouraud’s dextrose agar and cfu determined 24 h after incubation at 35°C.

**C. glabrata or C. albicans challenge of prophylactically treated animals**

The prophylactic studies were done in C57BL/6N mice as in our previous work. To assess prophylactic efficacy in a *C. glabrata* infection model, C57BL/6N mice were treated with different total doses of iv L-AmB and challenged at different times post-treatment (n = 7 per group): Group A = 24 h post-treatment, Group B = 3 days post-treatment and Group C = 7 days post-treatment. Group A mice were given 15 mg/kg once or 15 mg/kg twice (Monday and Thursday, 30 mg/kg total dose). Group B mice were given 15 mg/kg thrice (45 mg/kg total dose), four times (60 mg/kg total dose) or six times (90 mg/kg total dose) with injections on Mondays and Thursdays. Group C mice were given 15 mg/kg once, 15 mg/kg twice (30 mg/kg total dose), 15 mg/kg four times (60 mg/kg total dose) or 15 mg/kg six times (90 mg/kg total dose) with injections on Mondays and Thursdays. Control mice were untreated. Mice were immunosuppressed intraperitoneally with 100 mg/kg cyclophosphamide (Sigma) 3 days prior to fungal challenge and 75 mg/kg...
was then given every 3 days after that for the duration of the study (21 days). Mice were challenged intravenously via the tail vein with 2.0 × 10^6 C. glabrata either 24 h (Group A), 3 days (Group B) or 7 days (Group C) after treatment. Animals were monitored for morbidity (e.g. weight loss, activity level) for 21 days and then sacrificed. Their kidneys, the target of this severe, but non-lethal infection,22 which can disseminate to the spleen,23 were collected, weighed and homogenized in 1.0 mL of PBS using a 7 mm diameter probe on a Tissue Tearor (Biospec Products Inc.). If animals became moribund prior to the end of the study, they were sacrificed and their tissues similarly processed. Following serial dilutions of the tissue homogenates, 0.2 mL aliquots of each dilution were plated in duplicate on Sabouraud dextrose agar plates and incubated at 35°C for 24 h to determine cfu per gram of kidneys; the diluting procedures eliminated any effect on cfu due to drug carryover. The cfu for each treatment group was expressed as the median and range log_{10} cfu for that group of mice. The lower limit of detection in this assay was 15 cfu/g.

In the present study, we also tested the efficacy of prophylactic L-AmB in C57BL/6N mice (n = 30/group/time point) challenged with C. albicans. The mice were prophylactically treated with a cumulative iv dose of 60 mg/kg L-AmB, using two different dosing regimens to deliver the same total amount of drug: once per week at 15 mg/kg for 4 weeks or five times per week on Monday through Friday at 2.4 mg/kg for 5 weeks. Three weeks (n = 5 per group) and 6 weeks (n = 5 per group) after the last prophylactic dose, mice were sacrificed and their kidneys and spleens removed, weighed and frozen for subsequent bioassay of amphotericin B concentration. The remaining mice in each treatment group (n = 20) were challenged with C. albicans either 3 weeks (n = 10 per group) or 6 weeks (n = 10 per group) after the last dose of L-AmB. Mice were immunosuppressed with cyclophosphamide as described above. At the time of challenge, immunosuppressed mice were injected via the tail vein with ~0.97–1.22 × 10^5 blastocandin. Control infected mice were treated with comparable volumes of 5% dextrose (DSW) once per week for 4 weeks. Animals were monitored for morbidity (e.g. weight loss, activity level) for 7 days post-challenge, sacrificed on day 7 and both kidneys as well as the spleen removed from each mouse and processed as described above. The lower limit of detection in this assay was 15 cfu/g.

Statistical analysis
Tissue drug concentrations (µg/g) and tissue fungal burdens (cfu/g) were analysed by using GraphPad Prism, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). A one-way ANOVA was applied to compare the control with all groups in each experiment, and where differences occurred, a two-tailed Mann–Whitney test was done between paired groups. A P value of ≤0.05 was considered significant.

Results
Biodistribution of drug to tissues
To initiate the investigations, we examined the amount of L-AmB in the kidneys, lungs, liver and spleen 1 and 2 weeks after treatment with different total L-AmB doses. DBA/2 mice were treated with 15 mg/kg L-AmB thrice per week for 2 (90 mg/kg total dose), 3 (135 mg/kg total dose), 4 (180 mg/kg total dose) or 5 weeks (225 mg/kg total dose). Overall, based on µg/g tissue, the order of drug accumulation in the tissues was as follows: spleen > liver > kidneys > lungs (Figure 1).

If the animals received 135, 180 or 225 mg/kg cumulative doses, the drug levels in the kidneys were not significantly different from one another (P > 0.22, in all cases) (Figure 1a). Furthermore, the drug levels in the kidneys did not drop significantly between weeks 1 and 2 post-treatment (P > 0.22, in all cases). The 90 mg/kg dosing regimen, however, gave significantly lower drug levels in the kidneys at 1 week post-injection compared with the other dosing regimens (P < 0.032, in all cases). In comparison, drug levels in the lungs decreased markedly between weeks 1 and 2 for the 135 (P = 0.008), 180 (P = 0.095) and 225 mg/kg (P = 0.016) treatments. With 90 mg/kg dosing, there was no detectable drug in the lungs except for one animal at week 1. Only the highest dose showed detectable levels in the lungs in all mice in the group at 2 weeks post-treatment (Figure 1b). In the spleen at 1 week post-treatment, all dosing regimens, including the 90 mg/kg dose, produced similar mean drug levels (mean = 239–283 µg/g) (P > 0.69, in all cases). By 2 weeks, the drug levels had decreased, but the amount in the spleen was still not significantly different among the drug regimens (mean = 171–222 µg/g) (P > 0.40, in all cases) (Figure 1c). In the liver, drug concentrations were also not significantly different for the various dosing regimens (except for 90 versus 135 mg/kg at 1 week), but unlike the spleen, by 2 weeks, the drug levels had increased in this organ compared with the levels at 1 week (Figure 1d).

In the subsequent experiment, we used total doses of 180 and 225 mg/kg to examine the tissue drug levels at 12, 24 and 48 h post-treatment (Figure 2). Dosing schedules were the same as those described above. The drug levels did not drop significantly over 48 h when a total dose of 225 mg/kg was administered, except in the kidneys (P = 0.032), but the drug levels did drop in the kidneys (P = 0.056) and the spleen (P = 0.032) between 12 and 48 h when the mice were given a 180 mg/kg total dose. Comparison of the mean tissue drug levels in mice given a total dose of 225 mg/kg at 48 h (Figure 2) and 1 week (Figure 1) post-treatment showed a decrease of 24.6% in the spleen (375.1–282.7 µg/g), 37% in the kidneys (41.91–26.27 µg/g), 42.6% in the liver (82.49–47.35 µg/g) and 60% in the lungs (21.35–8.64 µg/g).

In another experiment, drug levels in the serum were examined over a period of 24 h following treatment with a total dose of 225 mg/kg. The levels in the serum peaked at 0.5 h (28.95 µg/mL) and by 2 h decreased by about half (13.55 µg/mL). The levels remained about the same for up to 12 h (4 h = 11.3 µg/mL, 8 h = 8.13 µg/mL, 12 h = 12.1 µg/mL) and then continued to decrease to a low level (1.0 µg/mL) by 24 h. Thus, the serum drug levels at 24 h post-treatment probably did not contribute significantly to the drug concentrations in the tissues at this time point.

Immunohistochemical localization of amphotericin B in tissues
To determine where the amphotericin B localized in each of the tissues following L-AmB treatment, an immunohistochemical procedure utilizing an anti-amphotericin B antibody was developed. Although the highest total dose of drug (225 mg/kg L-AmB) was used to try to ensure visualization of the drug
within different cells, the assay was unable to detect amphotericin B in the lungs at 3 and 7 days post-treatment in the uninfected animals. Drug concentration in the lungs as determined by our bioassay using this same dosing regimen was between 20 (2 days post-treatment) and 9 $\mu$g/g (7 days post-treatment). This indicated that the lower limit of sensitivity of the immunohistochemical assay in the uninfected mice was probably slightly above 20 $\mu$g/g. In comparison, positive amphotericin B immunostaining could be detected in the uninfected mouse kidneys, liver and spleen up to at least 1 week post-treatment (Figures 3 and 4). In the kidneys, positive amphotericin B staining was identified in tubular epithelium, primarily in the convoluted tubules, and in rare individual cells within the supporting interstitial stroma. Amphotericin B was not detected in the glomeruli. The tubular epithelial cells had granular cytoplasmic staining (Figures 3a and 3b). Positive staining cells within the interstitium had variable morphological features, with some cells morphologically similar to macrophages and others more closely resembling endothelial cells (Figure 3c). In the liver and spleen, the amphotericin B localized primarily within macrophages, i.e. Kupffer cells of the liver (Figure 4a and b) and red pulp macrophages of the spleen (Figure 4c and d). The increased number of macrophages in the liver and spleen of L-AmB-treated animals24 likely accounted for the elevated levels of amphotericin B delivered into and maintained in these tissues (Figures 1 and 2).

Figure 1. Concentrations of amphotericin B in $\mu$g/g (median ± range) determined by bioassay in (a) kidney, (b) lung, (c) spleen and (d) liver of DBA/2 mice ($n = 5$ per group) taken at 1 week (filled symbols) and 2 weeks (open symbols) after the final iv treatment of 15 mg/kg L-AmB. Cumulative doses of 90, 135, 180 or 225 mg/kg L-AmB were administered by intermittent iv injections of 15 mg/kg L-AmB/treatment on Mondays, Wednesdays and Fridays (90 mg/kg = 2 weeks of treatment; 135 mg/kg = 3 weeks of treatment; 180 mg/kg = 4 weeks of treatment; 225 mg/kg = 5 weeks of treatment). In kidneys at week 1: 90 mg/kg versus other dosing regimens, $P < 0.032$ in all cases. In lungs, week 1 versus week 2: 135 mg/kg, $P = 0.008$; 180 mg/kg, $P = 0.095$; 225 mg/kg, $P = 0.016$. In liver at week 1: 90 versus 135 mg/kg, $P = 0.008$.

L-AmB prophylaxis of C. glabrata infection

With drug levels in the kidneys of 20–27 $\mu$g/g 1 week after L-AmB treatment, studies were done to determine whether the efficacy of prophylactic treatment with L-AmB would be altered by the time interval between the completion of prophylactic
treatment and challenge. Previous studies had shown that a single dose of L-AmB (5, 10 or 20 mg/kg) would significantly reduce the kidney cfu/g if it were given 1 week prior to systemic challenge with *C. albicans.* In the present study, we used a murine *C. glabrata* infection model that also targets to the kidneys. L-AmB prophylaxis with either 15 mg/kg given as a single dose or 30 mg/kg L-AmB given as two doses of 15 mg/kg, when completed 1 day prior to challenge, produced a significant reduction ($P < 0.001$) in fungal burden in the kidneys with no detectable fungus in three of seven (43%) and six of seven (86%) of the treated mice, respectively (Figure 5a). Fungal challenge at 3 days after 45, 60 or 90 mg/kg L-AmB prophylaxis was associated with the same significant reduction ($P < 0.001$) in kidney fungal burden and a dose-dependent increase in the number of kidneys with undetectable *C. glabrata* (two of seven (29%), four of seven (57%) and five of seven (71%), respectively (Figure 5b). If the challenge was delayed for 1 week following prophylaxis with a total dose of either 15, 30, 60 or 90 mg/kg L-AmB, there was still a significant reduction ($P < 0.01$) in the kidney fungal burden for all doses compared with the controls (Figure 5c).

**Figure 2.** Concentrations of amphotericin B in µg/g (median ± range) determined by bioassay in (a) kidney, (b) lung, (c) spleen and (d) liver of DBA/2 mice (n = 5 per group) taken at 12, 24 or 48 h after the final iv treatment of 15 mg/kg L-AmB. Groups were treated with 15 mg/kg L-AmB on Mondays, Wednesdays and Fridays for 4 weeks (180 mg/kg, cumulative dose, open symbols) or 5 weeks (225 mg/kg, cumulative dose, filled symbols). 12 versus 48 h at 180 mg/kg in kidneys, $P = 0.056$, and in spleens, $P = 0.032$; 12 h versus 48 h at 225 mg/kg in kidneys, $P = 0.032$.

**Figure 3.** Kidney, 3 days (a) and 7 days (b) post-treatment. Renal tubular epithelium shows granular cytoplasmic staining for amphotericin B. Glomeruli are devoid of staining. Bar = 30 µm. (c) Kidney, 3 days post-treatment. Scattered cells within interstitial connective tissue stroma show cytoplasmic staining for amphotericin B, presumptive macrophages (arrows) and endothelial cells (arrowheads). Bar = 30 µm.
L-AmB dosing, accumulation and prophylaxis

Figure 4. Liver, 3 days (a) and 7 days (b) post-treatment. Kupffer cells show immunopositive cytoplasmic staining for amphotericin B (arrows). Bar = 30 μm. Spleen, 3 days (c) and 7 days (d) post-treatment. Red pulp macrophages show immunopositive cytoplasmic staining for amphotericin B (arrows). Bar = 30 μm.

L-AmB in kidneys and spleen 3–6 weeks post-treatment

Our data showed that L-AmB could be retained in the kidney and spleen tissues for up to 2 weeks post-treatment, and we wanted to determine whether L-AmB would still be present in the kidneys and spleen at 3 and 6 weeks post-treatment. We also wanted to investigate whether a daily (2.4 mg/kg five times per week for 5 weeks) or intermittent dosing (15 mg/kg once per week for 4 weeks) regimen, which delivered the same total amount of drug (60 mg/kg), would alter drug accumulation in these tissues at either or both time points.

Examination of drug levels in the spleen between 3 and 6 weeks showed close to a 67% decrease from 295 to 102 μg/g (or 265–116 μg/g) (P = 0.008 for both comparisons) whether the drug was given daily or intermittently (Figure 6b). The spleen drug levels at each time point were also the same for both dosing regimens (daily versus intermittent). In comparison, regardless of whether the drug had been given daily or intermittently (Figure 6a), the concentration of drug in the kidneys between 3 and 6 weeks did not decrease, and by 6 weeks there was an even significant increase in the drug level with daily dosing (P = 0.008). At 3 weeks, both daily and intermittent dosing regimens produced the same concentration of drug in the kidneys (P = 0.55).

L-AmB prophylaxis of C. albicans infection

To evaluate the prophylactic efficacy of L-AmB at 3 and 6 weeks post-treatment, mice were systemically challenged with C. albicans and monitored for survival and cfu/g in their kidneys and spleens. All of the mice survived to the end of the study (day 7 post-challenge), and their kidneys and spleens were collected for assessment of fungal burden. At both challenge time points, there was a significant reduction of ~2 logs in kidney cfu of drug-treated animals compared with the control mice (P < 0.0001), and the reduction was the same whether the drug had been given as daily or intermittent dosing (Figure 7a). In comparison, in the spleens, there was a significantly lower fungal burden with the intermittent dosing at 3 weeks (P < 0.02) but not at 6 weeks (P = 0.12) when the two dosing regimens were compared (Figure 7b). Comparison of dosing regimens with the control showed significant reductions in cfu in the spleen with both regimens at 3 weeks (P = 0.005 for the daily dosing and P < 0.0001 for the intermittent dosing) and at 6 weeks (P = 0.05 for the daily dosing and P < 0.0001 for the intermittent dosing).

Discussion

Previous investigators have shown that the peak serum level/MIC is a reasonable predictor of the antifungal efficacy of L-AmB25 or amphotericin B26 when the drug is given within 2–4 h following challenge with C. albicans. Their observations suggested that L-AmB as well as amphotericin B had concentration-dependent antifungal activity in these models. Andes et al.2 also demonstrated that an understanding of the antifungal potency differences between amphotericin B and the lipid formulations of amphotericin B benefited by including both serum and tissue pharmacokinetics. To further explore the pharmacodynamics of L-AmB, we focused on the drug accumulation and retention of amphotericin B in various tissues (i.e. lungs, kidneys, liver and spleen) for extended time periods (1–6 weeks) following multiple iv L-AmB dosing.

Amphotericin B levels given as L-AmB were maintained above the MIC for many pathogenic fungi (0.12–2.0 μg/mL)27,28 for as long as 6 weeks post-treatment. To determine whether the L-AmB in these tissues 1–6 weeks after treatment remained bioactive, we challenged the mice with C. albicans or C. glabrata and monitored the fungal burden in the kidneys and/or spleen. In these prophylaxis models, the fungal burden was reduced by several 1000-fold or was undetectable within the target tissues of many of the mice. It was reported by Andes et al.2 that L-AmB binding to the kidneys, lungs and liver ranged from 0% to 7% compared with 68% to 91% binding for amphotericin B deoxycholate, and this lack of L-AmB tissue binding could help to explain the sustained fungicidal activity of L-AmB in the tissues. In addition, we demonstrated in the C. glabrata model that prophylaxis could be achieved with only once or twice a week dosing rather than the daily dosing, which is the most frequently used clinical regimen for other drugs such as fluconazole,29 posaconazole30 and micafungin.31 Recent studies in an animal model have indicated that micafungin could be used once weekly as prophylaxis.32 Investigators have reported on the successful prophylactic use of L-AmB for other animal infections, such as Histoplasma capsulatum18 and A. fumigatus,33 and the combination of prophylactic and therapeutic treatment of animals for pulmonary aspergillosis34 and systemic fusariosis.35 The retention and sustained bioactivity of L-AmB is probably also related to the observation by Leenders et al.36 that L-AmB prevented dissemination of A. fumigatus infection from the lungs to the spleen and liver in a rat pulmonary aspergillosis model. The sustained tissue levels of L-AmB may also enhance the antifungal effects of sequential therapy when L-AmB is used
with another antifungal agent, such as an echinocandin or a triazole.

Although elevated levels of L-AmB were detected in the kidneys, this formulation of amphotericin B has significantly less nephrotoxicity compared with other formulations.\textsuperscript{4,5,7,8,37} The immunohistochemical results in our study showed that the amphotericin B from the L-AmB localized in the cytoplasm of the renal tubular epithelial cells. It was also seen in the stroma of the kidneys, within cells that appeared to be macrophages and endothelial cells. The cells of these kidneys looked normal, and the lack of cellular toxicity suggested that the L-AmB was not toxic for the kidney cells. The lack of L-AmB toxicity for Langerhans cells has been reported previously.\textsuperscript{38} These observations suggest that amphotericin B delivered as L-AmB to the kidneys may remain associated with the lipids of the liposome, reducing the drug’s toxic interactions with mammalian cell membranes.

The clearance of L-AmB from the lungs of uninfected mice was faster than that from the kidneys, spleen or liver, although mean levels of 15.6 \( \mu \text{g/g} \), which are \( \sim 7.8 \) to 15 times above the MIC for many pathogenic \textit{Aspergillus} species that cause pulmonary infection, were still detected in the lungs 2 days after dosing. Even after 1 week, there was a mean of 4.8 \( \mu \text{g/g} \) of L-AmB in the lungs. The MIC\textsubscript{90s} for \textit{A. fumigatus}, \textit{Aspergillus flavus},

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Log\textsubscript{10} cfu/g kidney from prophylactically treated, immunosuppressed C57BL/6N mice (\( n = 7 \) per group) challenged intravenously with \( 2.0 \times 10^6 \) \textit{C. glabrata}/mouse. (a) Mice were treated once or twice with 15 mg/kg L-AmB and challenged with \textit{C. glabrata} 24 h post-treatment. (b) Mice were treated with 15 mg/kg L-AmB on Mondays and Thursdays for a total of three injections (45 mg/kg, cumulative dose), or they were treated on Mondays and Thursdays for 2 weeks (60 mg/kg, cumulative dose) or 3 weeks (90 mg/kg, cumulative dose), and then 3 days after the final treatment challenged with \textit{C. glabrata}. (c) Mice were treated with 15 mg/kg L-AmB once or 15 mg/kg L-AmB on Mondays and Thursdays for 1 week (30 mg/kg cumulative dose), 2 weeks (60 mg/kg cumulative dose) or 3 weeks (90 mg/kg cumulative dose), and then 7 days after the final treatment challenged with \textit{C. glabrata}. Horizontal bars represent the median value for each group. (a) Treatments versus control, \( P < 0.001 \) in all cases; (b) treatments versus control, \( P < 0.001 \) in all cases; (c) 15 mg/kg versus control, \( P = 0.007 \); 30 mg/kg versus control, \( P < 0.001 \); 60 mg/kg versus control, \( P < 0.001 \); 90 mg/kg versus control, \( P = 0.001 \). Tx, treatment.}
\end{figure}
Aspergillus niger and Aspergillus terreus range from 1 to 2 μg/mL. The lack of detectable L-AmB in the cells of the uninfected lungs between 3 and 7 days post-treatment using the immunohistochemical technique was most likely a result of the limited sensitivity (>20 μg/mL) of this assay compared with the bioassay (0.03 μg/mL). Amphotericin B from L-AmB, however, was detected in the lungs of A. fumigatus-infected mice by an immunofluorescent assay, suggesting that either the immunofluorescent assay was more sensitive than the immunohistochemical assay that we used or possibly that more drug was delivered to the lungs when it was infected with A. fumigatus. Preliminary data (J. Schwartz, unpublished results) has shown that the immunohistochemical assay can be used to detect L-AmB in the cells of A. fumigatus-infected lungs at 2 days post-infection in animals that received a total cumulative dose of 80 mg/kg.

The concentration of L-AmB in the spleen peaked at ~500 μg/g, with a slow decrease over time, and drug levels remained above 150 μg/g for as long as 2 weeks post-treatment.
In the liver, the concentration of L-AmB increased over 2 weeks from \( \sim 40 \) to \( 60 \mu g/g \) or higher. Considering that the liver is \( \sim 10 \) times the size of the spleen, it is clear that the liver as a whole retained more drug than any other organ. The immunohistochemical data demonstrated that the L-AmB in both the spleen and liver were present in the tissue macrophages. These cells have a high capacity to take up particulate material,\(^{41,42}\) and this probably helps to explain the high levels of drug retained in these tissues.

Several studies have reported autopsy data on L-AmB distribution in human tissues.\(^{9,43,44}\) Despite the variability in cumulative dosing, the studies consistently reported that the highest amphotericin B concentrations were found in liver (103 \( \pm 69 \mu g/g \)) and spleen (60.3 \( \pm 29.8 \mu g/g \)), whereas lesser amounts of drug accumulated in the kidney (11.9 \( \pm 12.8 \mu g/g \)) and lung (11.6 \( \pm 7.7 \mu g/g \)).\(^{44}\) Interestingly, both Heinemann et al.\(^{43}\) and Vogelsinger et al.\(^{44}\) reported that individual patients with impaired liver function had relatively high lung concentrations of drug, suggesting that when greater levels of L-AmB remain in circulation, more drug will accumulate in the lungs. Also, at the highest cumulative dose of L-AmB administered, increased concentrations of amphotericin B were detected in lung tissue of the patients, whereas liver and spleen concentrations remained relatively unchanged with increased dosing, thus suggesting that as the reticuloendothelial system (RES) becomes saturated, more drug is available to accumulate in the lungs. Our results from the biodistribution studies in mice are consistent with the existing human data. For example, with a cumulative dose of 180 mg/kg L-AmB 1 week post-treatment, we observed higher drug concentrations in the liver (39 \( \mu g/g \)) and spleen (252 \( \mu g/g \)), whereas lower accumulations were found in the kidney (28 \( \mu g/g \)) and lung (5.0 \( \mu g/g \)).

The pre-clinical data reported in the present study underscore the importance of including drug tissue levels, along with serum pharmacokinetics, to obtain a better understanding of L-AmB efficacy in both the prophylactic and therapeutic setting. Further work to compare drug concentrations in infected and uninfected animals could help to determine how the infection impacts drug accumulation and distribution. These types of investigations may be useful in helping to develop appropriate clinical L-AmB dosing regimens for various types of fungal infections and treatment indications.

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