High-dose induction liposomal amphotericin B followed by de-escalation is effective in experimental Aspergillus terreus pneumonia

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Objectives: Aspergillus terreus is considered to be resistant to amphotericin B (AMB). However, it is unknown whether higher daily doses of liposomal AMB (L-AMB) can overcome this resistance in vivo. We evaluated the efficacy and total lung homogenate AMB concentrations of escalating intravenous doses of L-AMB (3–20 mg/kg daily) versus an induction-de-escalation dosing strategy (10 mg/kg/day × 3 days, then 3 mg/kg/day) in an experimental neutropenic murine model of A. terreus pneumonia.

Methods: BALB/c mice were rendered neutropenic with cyclophosphamide and administered cortisone acetate prior to intranasal inoculation (3.5 × 10^6 conidia) with A. terreus (Etest MIC 8 mg/L). Mice were then treated with L-AMB regimens for 5–7 days. The efficacy was assessed by animal survival and quantitative PCR lung fungal burden. Total AMB lung homogenate concentrations were determined by HPLC.

Results: Compared with untreated controls, 10 mg/kg/day L-AMB prolonged survival (mean 7 versus 3–4 days, P < 0.003) and reduced A. terreus lung fungal burden (median log10 conidial DNA 5.0 versus 6.7, P < 0.05). Daily L-AMB regimens > 10 mg/kg/day were associated with poorer survival and higher lung fungal burden. The induction-de-escalation strategy of 10 mg/kg/day × 3 days followed by 3 mg/kg/day was as effective as 10 mg/kg daily dosing, and resulted in higher mean AMB lung homogenate concentrations compared with a continuous 10 mg/kg regimen (23.2 ± 6.7 versus 16.4 ± 4.4 µg/g, P = 0.09).

Conclusions: A high-dose induction-de-escalation L-AMB dosing strategy was an effective treatment for experimental A. terreus pneumonia in neutropenic mice.

Keywords: pharmacokinetics, pharmacodynamics, invasive aspergillosis, murine infection model

Introduction

Aspergillus terreus is a ubiquitous soil saprophyte that accounts for 1%–13% of all culture-documented cases of aspergillosis, although the prevalence of this species can approach 30% in some hospitals. A. terreus is considered to be intrinsically resistant to amphotericin B (AMB), even though the mechanisms underlying this resistance are poorly understood. Diminished cell membrane ergosterol, differences in the fungal cell wall and a higher basal catalase production have been proposed as mechanisms underlying AMB resistance in A. terreus, but have not been conclusively demonstrated to cause AMB treatment failures in vivo.

Similarly, it is not known whether intrinsic AMB resistance in A. terreus can be overcome with higher AMB exposures in vivo. Based on data from previous experimental models, we hypothesized that A. terreus pneumonia may be treatable with high daily doses of liposomal AMB (L-AMB) (>5 mg/kg/day) or induction-de-escalation dosing approaches that rapidly load lung tissue with AMB to a level that surpasses the reported MIC of 4–8 mg/L for most clinical isolates. Herein, we describe our testing of this hypothesis using a neutropenic murine model of A. terreus pneumonia.

Methods

Study drugs

Cortisone acetate and cyclophosphamide were obtained from Sigma-Aldrich (St Louis, MO, USA). The human clinical formulation of L-AMB (AmBisome®) was obtained from the hospital pharmacy and diluted in sterile 5% dextrose water immediately prior to administration in animals.
Inoculum preparation

A clinical isolate of A. terreus was selected for testing and prepared as an infecting inoculum as previously described.6 The AMB MIC was 0.5 mg/L by the Clinical Standards Laboratory Institute broth microdilution M38-A2 method8 and 8 mg/L by AMB epsilometer strips (bioMérieux, Durham, NC, USA).

Murine infection model

Eight-week-old female BALB/c mice (18–25 g, Charles River Laboratories, Houston, TX, USA) were used for all experiments. Mice were housed in HEPA filtration cage systems and had access to sterile food and water ad libitum. All mice were cared for in accordance with the highest standards for humane and ethical care, the experimental procedures were approved by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Animals were immunosuppressed with intraperitoneal (ip) injections of cyclophosphamide (150 mg/kg) at 4 days and 1 day prior to infection, with an additional cyclophosphamide dose at day +2 to maintain neutropenia for 5 days.10 A single 300 mg/kg ip dose of cortisone acetate suspension prepared in PBS +0.2% Tween 20 was also administered 1 day prior to infection, to suppress pulmonary macrophage function prior to animal inoculation.

On the day of infection, animals were rendered unconscious in 6% isoflurane:oxygen using a small-animal anaesthesia chamber before a 3.5 µL droplet containing 3.5 × 108 A. terreus conidia was slowly introduced using a micropipette into the nares of the unconscious breathing mouse held in an upright position. After normal breathing resumed, animals were transferred back to cages and antifungal therapy was started 12 h later.

Antifungal treatment and study endpoints

After infection, immunosuppressed mice (n = 12–15 per group) were administered L-AMB intravenous injections through the lateral tail vein at 3, 10, 15 or 20 mg/kg daily for 5 days. The 15 and 20 mg/kg doses were split into two injections, 8 h apart to minimize the risk of acute toxicity. Control animals were administered intravenous 5% dextrose water alone. For the induction-de-escalation dosing strategy, mice were initially treated with intravenous L-AMB daily doses of 10 mg/kg for 3 days and then randomly grouped to continue receiving the daily 10 mg/kg dose or reduced dose of 3 mg/kg/day (de-escalation strategy) until day +7.

The efficacy of each L-AMB dosing regimen was assessed by animal survival at day +5 (daily dosing regimens) or at day +7 (induction-de-escalation strategy). The animals that appeared moribund (ruffled fur, difficulty breathing, decreased or altered mobility, hypothermia) or survived until the end of treatment were euthanized by CO2 asphyxiation and the lungs were aseptically removed, weighed and homogenized for analysis of the A. terreus fungal burden by quantitative real-time PCR of the A. terreus 18S rRNA gene using previously described methods.11

Total lung concentrations of AMB were also determined in each tissue homogenate analysed by PCR using an ultra-HPLC assay that has been previously reported.6,7 The calibration curve was linear over a range of 0.25–10 μg/g in tissue. The mean inter- and intra-assay coefficients of variation over the standard curve were <10%. The lower limit of accurately detectable AMB in the tissue was 0.25 μg/g.

Results

Efficacy of daily-dose L-AMB regimens

Neutropenic mice receiving an intranasal inoculation with 3.5 × 106 A. terreus conidia developed rapidly progressing bronchopulmonary infection that was 100% fatal by day +3 in control animals (Figure 1a). Compared with control animals, intravenous treatment with 3 mg/kg L-AMB improved survival to 40% by day +5 (P = 0.008). Daily intravenous doses of 10 mg/kg L-AMB further improved survival to 70% (P = 0.005 versus control), but were not statistically different from 3 mg/kg/day L-AMB (P = 0.28). Interestingly, daily L-AMB doses >10 mg/kg were associated with poorer survival compared with L-AMB dosed at 3–10 mg/kg (Figure 1a), possibility related to drug (nephro)toxicity (some mice lost 15%–20% of body weight) and a requirement for early euthanization because of CNS adverse effects, which were likely to be infection related. All other treatment regimens were associated with weight loss of 5%–10% (1–2 g) in infected animals during the course of the experiment.

The pulmonary A. terreus fungal burden paralleled survival differences that were observed among the treatment groups (Figure 1b). The median A. terreus conidial equivalent DNA concentrations per lung averaged log10 6.7 in control animals, log10 6.0 in animals treated at 3 mg/kg/day (P > 0.05 versus control) and log10 5.0 in animals treated with the 10 mg/kg/day dose. Consistent with the survival data, animals treated with 15 mg/kg daily or 20 mg/kg daily of L-AMB had a higher median A. terreus lung fungal burden (log10 6.7 and 5.9, respectively) that was not significantly different from untreated controls.

Based on the results of the daily-dose experiments, we hypothesized that an L-AMB regimen of induction dosing with 10 mg/kg daily for 3 days followed by de-escalation to 3 mg/kg daily may be as effective as 10 mg/kg daily dosing for experimental A. terreus pneumonia. When these treatment regimens were compared with the infected control (Figure 1c), we found that the induction-de-escalation L-AMB dosing strategy achieved the highest day +7 survival rates compared with the 10 mg/kg daily dosing regimen (100% versus 80%, P = 0.11) and a similar median lung fungal burden (log10 5.0 versus 5.0, respectively; Figure 1b).

AMB lung homogenate concentrations

The administration of higher daily L-AMB doses in infected mice resulted in near-linear increases in total lung AMB tissue concentrations over the studied dose range (Figure 1d). The mean day +5 lung concentrations increased from 1.8 ± 0.48 μg/g at 3 mg/kg daily L-AMB dosing to 12.5 ± 1.76 μg/g at 20 mg/kg daily. Mean day +7 lung tissue concentrations of AMB were higher in animals that received the induction-de-escalation dosing of L-AMB, compared with animals that remained on the 10 mg/kg daily dosing regimen (23.2 ± 6.7 versus 16.4 ± 4.4 μg/g, P = 0.09). Tissue homogenate concentrations with both regimens surpassed the MIC of the A. terreus test isolate (8 mg/L).

Discussion

L-AMB has complex, dose-dependent, non-linear pharmacokinetics in rodents, canines and humans.12–15 At higher doses (i.e. >5 mg/kg/day), the rate of AMB release from the liposome carrier in plasma decreases, reticular endothelial cell-mediated clearance/distribution is saturated12–15 and patterns of tissue drug distribution change depending on dose, duration of therapy and probably the inflammatory state and severity of
infection in the host.\textsuperscript{12–15} As a result, it is difficult to predict whether a higher daily dosage of L-AMB will result in increased AMB tissue concentrations in the lung that would be theoretically important for overcoming the relative resistance of \emph{A. terreus}.

Two previous studies have examined higher-dose AMB deoxycholate\textsuperscript{16} or L-AMB\textsuperscript{17} treatment regimens in experimental \emph{A. terreus} disseminated infection. Both studies utilized an intravenous challenge model of \emph{A. terreus} infection and found no evidence of efficacy with the higher-dose regimens. Our study differs in that we utilized a sinopulmonary infection model that allowed us to focus on dosing efficacy in a single-target organ of infection. We found that, in contrast to previous reports, a 10 mg/kg daily L-AMB dose regimen or an induction-de-escalation strategy of 10 mg/kg $\rightarrow$ 3 mg/kg daily prolonged survival in neutropenic mice and significantly reduced lung fungal burden versus infected controls. Interestingly, the induction-de-escalation treatment regimen also produced the highest concentrations of total AMB in crude lung homogenate analysed by HPLC, suggesting a possible saturation in pulmonary uptake with higher doses; although it is impossible to state with certainty whether this actually reflects high active drug concentrations in the lung tissue.\textsuperscript{18}

Our experimental findings challenge the conventional wisdom that \emph{A. terreus} is uniformly AMB resistant and suggest that some \emph{A. terreus} isolates may be ‘treatable’ with L-AMB, provided the dosing regimen can optimize drug delivery to the site of infection while minimizing drug delivery to the kidney. An induction-de-escalation dosing strategy for L-AMB may be one approach

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**Figure 1.** Dose-dependent treatment efficacy of L-AMB in a murine model of \emph{A. terreus} pneumonia. (a) Survival of mice infected with \emph{A. terreus} and treated with various daily intravenous dosages of L-AMB. Each group contains 12–15 mice. Groups were compared using the Mantel–Cox (log-rank) test; *P $<$ 0.05 (considered statistically significant). (b) Dose-dependent patterns of tissue fungal burden reduction in mice with \emph{A. terreus} pneumonia determined by quantitative real-time PCR. CE DNA/lung = conidial equivalent DNA/lung interpolated from a standard curve. Groups were compared using the Kruskal–Wallis test with Dunn’s post-test for multiple comparisons. P $<$ 0.05 was considered statistically significant. Horizontal lines represent the median lung fungal burdens of the treatment groups. (c) Survival of mice treated with L-AMB at 10 mg/kg/day or a de-escalated dose of 10 mg/kg/day $\times$ 3 days, then 3 mg/kg/day. Each group contains 12–15 mice. Experimental groups were compared in a similar manner to those in (a). (d) Total AMB concentrations in tissue homogenate determined by ultra-HPLC.
for salvaging the activity of this broad-spectrum antifungal for A. terreus and should be further investigated for other treatment-refractory moulds and in other sites of infection.

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Transparency declarations

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