In vitro pharmacodynamics of rapid versus continuous infusion of amphotericin B deoxycholate against Candida species in the presence of human serum albumin

Russell E. Lewis1,2*, Nathan P. Wiederhold3,4, Randall A. Prince1,2 and Dimitrios P. Kontoyiannis1,2

1The University of Houston College of Pharmacy, Houston, TX, USA; 2The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; 3The University of Texas at Austin, College of Pharmacy, Austin, TX, USA; 4The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

Received 23 July 2005; returned 5 October 2005; revised 12 November 2005; accepted 1 December 2005

Background: Recent open label studies have suggested that dosing amphotericin B (AMB) by continuous infusion (CI) may reduce drug-associated infusion reactions and nephrotoxicity. In vitro and in vivo pharmacodynamic (PD) data, however, do not consistently support the concept of CI dosing based on the concentration-dependent activity of this agent and in vitro studies with AMB rarely account for the drug’s high degree of protein binding. Therefore, we compared the PD activity of simulated continuous versus rapid infusion strategies of AMB in killing of AMB-susceptible and -resistant Candida species using an in vitro pharmacodynamic model.

Methods: Time–kill curves were performed with Candida albicans (Etest MIC 0.38 mg/L) and Candida lusitaniae (MIC 1.5 mg/L) at AMB concentrations between 0 and 16 mg/L in the absence and presence of 4 and 8% human serum albumin (HSA). A one-compartment in vitro pharmacodynamic model was used to simulate the steady-state PK parameters of bolus and CI AMB.

Results: The fungicidal activity of AMB was attenuated by the presence of HSA for both Candida species tested. The EC50 for each isolate significantly increased in the presence of 4% HSA (P < 0.05), and fungicidal activity was completely abated for C. lusitaniae when HSA concentrations were increased to 8%. No substantial differences in the rate or extent of AMB killing were observed between rapid infusion or CI dosing and neither regimen produced fungicidal activity in the presence of HSA.

Conclusions: The presence of HSA changes the in vitro PD of AMB. In our model, CI and rapid infusion dosing of AMB exhibited similar activity when attempts were made to correct for protein binding that is likely to occur in vivo.

Keywords: Candida albicans, Candida lusitaniae, protein binding

Introduction

Despite promising reports demonstrating reduced nephrotoxicity and infusion-related reactions,1–6 the practice of administering amphotericin B deoxycholate by continuous infusion (CI) to reduce infusion and drug-induced nephrotoxicity has not become a standard of care in the treatment of life-threatening mycoses. Concerns that have limited the widespread adoption of continuous infusion dosing include (i) the lack comparative data from prospective randomized trials supporting the efficacy of 24 h infusion of amphotericin B for proven mycoses; (ii) the necessity of dedicating venous access in critically ill patients solely for the administration of amphotericin B, which is incompatible with numerous intravenous solutions; and (iii) data demonstrating that the concentration-dependent pharmacodynamics of amphotericin B are best optimized by infrequent administration of high daily dosages.7,8 Indeed, several in vitro and animal studies have supported the concept that amphotericin B exhibits concentration-dependent...
activity against susceptible yeast and moulds. However, dosage escalation studies of the lipid formulations in humans or animals have not been prospectively performed, and available data provide surprisingly little clear-cut evidence of a steep dose–response above currently recommended dosages.

Although a multitude of factors may contribute to the discordance of antifungal activity observed in vitro versus in vivo, the biopharmaceutical properties of amphotericin B may be especially relevant for interpretation of drug susceptibility and pharmacodynamics. Amphotericin B exhibits non-linear, concentration-dependent protein binding in plasma and tissues that is virtually unsaturable over a range of human drug exposures (0.6–65 mg/L). While the drug may be 95% protein bound at 0.6 mg/L, a 100-fold increase in drug concentrations does not increase the total amount of unbound (and presumably bioactive) drug. This unusual pattern of protein binding may be a result of the poor aqueous solubility of the drug at a neutral pH (i.e. the amphoteric properties of the drug).

In a series of experiments utilizing equilibrium dialysis to characterize patterns of amphotericin B protein binding in human plasma, Bekersky et al. estimated that the solubility of unbound amphotericin B in human plasma maximizes at 0.744 mg/L. Further increases in total drug concentration did not result in an increasing amount of unbound amphotericin B in human plasma. Of interest, this ‘solubility threshold’ is similar to peak concentrations maintained in the human plasma with continuously infused amphotericin B dosages of 0.6–0.8 mg/kg/day, but below peak concentrations (1.5–2.5 mg/L) achieved with conventional 4–6 h infusions at similar doses. It is possible that concentration-dependent changes in protein binding, which occur with different infusion rates, could change the rate of distribution of amphotericin B to various tissues such as the kidneys. From a pharmacodynamic perspective, if higher peaks above the maximal solubility threshold of amphotericin B do not result in a meaningful increase in non-protein-bound bioactive drug at the site of infection, then minimal differences would be expected in the rate and extent of fungal killing between rapid and continuously infused amphotericin B regimens.

Using this conceptual framework, we compared the in vitro pharmacodynamics of amphotericin B deoxycholate alone, and in the presence of human serum albumin (HSA), against Candida species with MICs below and above the proposed solubility threshold (744 ng/mL) of amphotericin B in human serum. We then evaluated killing rates of a bolus and continuously infused amphotericin B regimen using an in vitro pharmacodynamic model to simulate the polyene’s serum half-life in the absence and presence of HSA. We found that the addition of HSA at physiologically relevant concentrations (4% w/v or 4 g/dL, simulating normal human serum concentrations) significantly attenuated the pharmacodynamic profile of amphotericin B.

Pharmacodynamics of rapid versus continuous AMB infusions

Materials and methods

Antifungal preparation and growth medium

Amphotericin B deoxycholate solution 5 g/L was prepared fresh prior to each experiment by reconstituting a 10 mL vial of amphotericin B deoxycholate lyophilized powder (Pharma-Tek, Inc., Huntington, NY, USA) with sterile water. Drug stock was then diluted in RPMI 1640 growth medium (Sigma Chemicals, St Louis, MO, USA) buffered to a pH of 7.0 with 0.165 M MOPS + 2% glucose. In select experiments albumin was added to RPMI growth medium to a final concentration of 4 or 8% from a commercially available solution (25%) of pooled HSA (ZLB Bioplasma Inc., Glendale, CA, USA). Concentrations of albumin used for in vitro studies were selected on the basis of normal physiological concentration (4%) of serum albumin in humans (range 3.5–5 g/dL). A supra-physiological serum concentration of albumin (8%) was included to examine the potential for further reductions in amphotericin B fungicidal activity which could occur in other tissue sites or in the presence of inflammation.

Isolates and susceptibility testing

Candida albicans (ATCC 90028) and a clinical isolate of C. lusitaniae 324/4F recovered from the bloodstream of a non-neutropenic patient with sepsis on amphotericin B therapy were used for all testing. Isolates were subcultured twice on potato dextrose agar (Remel, Lenexa, KS, USA) prior to preparation of a standardized inoculum (0.5 McFarland turbidity standard corresponding to 1 × 10⁶ cfu/mL) according to the Clinical Laboratory Standards Institute (formally NCCLS) M27-A2 methods. Because broth microdilution methods have a limited ability to detect amphotericin B-resistant strains, Epsilometer strips (Etest, AB Biodisk, Solna, Sweden) were used to determine amphotericin B MICs on 1.5% RPMI agar according to the manufacturer’s instructions.

Time–kill studies

Time–kill studies of amphotericin B were performed with the C. albicans and C. lusitaniae isolates in (i) RPMI growth medium alone and (ii) growth medium containing 4% and 8% HSA using a modification of standardized methods. Briefly, a suspension of fungal cells standardized to 0.5 McFarland turbidity was diluted 1:10 in RPMI growth medium with or without albumin. The suspension was then diluted 1:2 in a similar growth medium with or without amphotericin B to final concentrations ranging between 0.0625 and 16 mg/L. At serial time-points after the addition of the drug (0, 2, 4, 8, 12 and 24 h), tubes were sampled (0.1 mL) and plated (0.05 mL) on potato dextrose agar using a spiral plater dispenser (Autoplate 4000, Spiral Biotech, Bethesda, MD, USA). Plates were then incubated for 48 h at 35°C and colonies were analysed using a computerized scanner for cfu enumeration (CASBA-4 Colony Image Analysis, Spiral Biotech). This sampling method can accurately quantify 50 cfu/mL with minimal or no antifungal carryover. Concentrations that showed evidence of amphotericin B carryover upon spiral plating (improved growth with dilution) were further diluted (1:10 or 1:100) and filtered through a 0.2 micron filter. The filters were washed with sterile saline, placed onto potato dextrose agar and incubated for 24 h at 37°C prior to cfu enumeration. All time–kill experiments were performed in triplicate.

Pharmacodynamic model

A previously described one-compartment in vitro pharmacodynamic model was used to simulate the serum pharmacokinetics of a bolus infusion of amphotericin B deoxycholate or a continuous infusion equivalent to plasma concentrations achieved with a 1 mg/kg/day dose in humans (Figure 2). Briefly, sterile drug-free RPMI growth medium ±% HSA was then pumped into the central compartment (250 mL) with a computerized peristaltic pump (Masterflex LS, Vernon Hills, IL, USA) at a fixed rate to simulate the 24 h steady-state serum half-life of amphotericin B. After the desired flow rate in the model was established, the central compartment was inoculated

289
with a suspension of *C. albicans* or *C. lusitaniae* to achieve a starting inoculum of 1–5×10^5 cfu/mL. The model was allowed in equilibrate for 30 min before the total daily dose of amphotericin B was administered as either a bolus infusion (target peak 2.4 mg/L) or a loading dose (target concentration 1 mg/L) followed by a continuous infusion delivered through a computerized infusion pump to maintain drug concentrations at 0.8–1 mg/L. The bolus infusion was redosed 24 h later at 50% of the initial dose to account for trough concentrations of amphotericin B. All experiments were run for 48 h. At serial time-points following antifungal dosing (0, 2, 4, 8, 12, 24, 36 and 48 h) samples were removed from the central compartment of the model and plated on potato dextrose agar for cfu enumeration as described previously. All experiments were performed in triplicate.

**Amphotericin B assay**

In select experiments performed with non-protein-containing growth medium in the pharmacodynamic model, samples (0.2 mL) were collected at serial time-points (0, 0.5, 2, 4, 8, 12, 24, 24.5, 30 and 48 h) from the central compartment and frozen at −80°C for verification of amphotericin B pharmacokinetics in the model. Drug concentrations were determined using a standard drug diffusion bioassay and the sensitive indicator organism *Candida kefyr* (ATCC 66028, Etest MIC 0.125 mg/L). Assays of the unknown samples from the model and the standard curve (0.25–8 mg/L) were prepared in triplicate with amphotericin B stock in RPMI growth medium. Pharmacokinetic constants were calculated via the non-linear least squares method (Stata Rel 8, College Station, TX, USA). The AUC was calculated by analysis of variance with Tukey’s test for multiple comparisons. For all comparisons, a *P* ≤ 0.05 was considered significant. All analysis of cfu plots was performed using the Graphpad software.

**Results**

**Susceptibility testing**

Median 48 h amphotericin B MICs determined by Etest were 0.38 mg/L for *C. albicans* 90028 and 1.5 mg/L for *C. lusitaniae* 324/4F. MICs of the isolates recovered from the pharmacodynamic model did not differ significantly from baseline MIC determinations.

**Time–kill studies**

Sigmoid inhibitory dose–response curves for amphotericin B alone and in the presence of 4% and 8% albumin against *C. albicans* and *C. lusitaniae* are presented in Figure 1. Time–kill studies revealed a significant attenuation of amphotericin B fungicidal activity in the presence of albumin. Significant increases in the EC50 of amphotericin B were observed in both *Candida* isolates when albumin was added to the culture medium. This effect was intensified at higher concentrations of HSA. For *C. albicans*, the EC50 increased from 0.5 mg/L (95% CI, 0.41–0.63) to 1.31 mg/L (1.05–1.62) at 4% albumin and 2.72 mg/L (2.16–3.45) at 8% albumin (*P* < 0.05). Minimum fungicidal concentrations of amphotericin B similarly increased from 2 to 6.90 mg/L and 12 mg/L at concentrations of 4 and 8% albumin, respectively (*P* < 0.001 versus no protein

**Figure 1.** Sigmoidal inhibitory dose–response curves of amphotericin B deoxycholate alone (open squares) and in the presence of 4% (closed squares) and 8% (closed circles) HSA against (a) *C. albicans* 90028 and (b) *C. lusitaniae* 324/4F. Data plots were generated from 24 h time–kill studies performed in triplicate. A four-parameter logistic regression model (Hill equation) was fitted without weighting to data to derive the EC50 use curve-fitting software.
for 4 and 8%). For *C. lusitaniae*, the EC50 increased from 0.36 mg/L (0.26–0.46) to 1.80 mg/L (1.50–2.20) at 4% albumin and to >16 mg/L at 8% albumin (*P* < 0.05). Surprisingly, the steepness of the concentration–effect curve for *C. lusitaniae* was steeper than that observed with *C. albicans* despite the decreased susceptibility of this isolate by agar-based Etest strip testing. This may be due in part to the slower growth rate of *C. lusitaniae* compared with *C. albicans* in RPMI medium. At 8% albumin, amphotericin B exhibited minimal fungistatic activity against *C. lusitaniae* even at concentrations that exceeded 10× MIC of the isolate. Amphotericin B fungicidal concentrations against *C. lusitaniae* increased from 0.5 to 4.8 mg/L and >16 mg/L in the presence of 4 and 8% albumin, respectively (*P* < 0.001 versus no protein for 4 and 8%).

**Amphotericin B pharmacokinetics**

Bioassay results of the standard curve were linear over the range of concentrations tested (0.25–8 mg/L). Intraday coefficients of variation ranged from 3 to 10.2%. The lower limit of detection for the assay was 0.25 mg/L. A plot of amphotericin B concentrations without protein interpolated from the standard curve over time for bolus and continuous infusion is presented in Figure 2. Actual drug concentrations and pharmacokinetic parameters in the model were close to predicted values, although the elimination rate of amphotericin B was greater than expected (*t*½ of bolus infusion 12–16 h), with a similar effect observed for the continuous infusion regimen that averaged concentrations 0.7–0.8 mg/L (Figure 2). The rapid elimination of amphotericin B, possibly due to drug degradation, is consistently seen in vitro pharmacodynamic models testing this drug.18,19 Bolus infusions produced a higher peak concentration (*C*max = 3.04 mg/L; *AUC*0–48 = 70.4 mg·h/L) compared with the continuous infusion regimen (*C*max = 1.01 mg/L; *AUC*0–48 = 35.7 mg·h/L). Importantly, both regimens maintained amphotericin B concentrations above the expected maximal unbound concentration of amphotericin B in protein of 744 ng/mL.

**Pharmacodynamic model**

As observed in the time–kill studies the addition of 4% HSA to the *in vitro* model markedly diminished the fungicidal activity of bolus and continuous infusion amphotericin B regimens against *C. albicans* and *C. lusitaniae* (Figure 3). Samples of control regimens in the model revealed a 1–1.5 log10 increase in cfu counts for both *C. albicans* and *C. lusitaniae* by 24 h that was unaffected by the presence of albumin. In contrast, without albumin both the bolus and the continuous infusion regimen produced fungicidal activity (3–4 log10 drop in cfu counts) within 24 h. The rate of
killing appeared to be somewhat more rapid with the bolus regimen in the absence of albumin, particularly for \(C. lusitaniae\) where the time to \(3 \log_{10}\) kill was \(\sim 8\) h for the bolus regimen versus \(14\) h for the continuous infusion regimen. However, these curves were not statistically different. Fungicidal activity for both regimens was completely lost against both \(C. albicans\) and \(C. lusitaniae\) in the presence of albumin. Fungicidal activity against \(C. albicans\) was reduced \(2–2.5 \log_{10}\) with the addition of \(4\%\) albumin \((P < 0.001)\) for both the bolus and continuous infusion regimens (Figure 3a). Although the bolus regimen appeared to be slightly more effective for \(C. albicans\) in the presence of albumin than the continuous infusion, differences in cfu/mL recovered from the model for the two regimens were not significantly different \((P > 0.05)\).

A similar abatement of fungicidal activity was observed for \(C. lusitaniae\) \((P < 0.001)\) (Figure 3b), where cfu killing curves were virtually identical for the bolus and continuous infusion regimens in the presence of \(4\%\) albumin.

**Discussion**

*In vitro*, amphotericin B exhibits rapid, concentration-dependent fungicidal activity against *Candida* species which is rarely matched by other antifungals.\(^8\) However, *in vivo*, amphotericin B has activity similar to an antifungal reported to have fungistatic activity, rarely sterilizes tissues and demonstrates minimal evidence of a steep dose–response curve.\(^10\) We hypothesized that the unusual biopharmaceutical properties of amphotericin B may contribute to the discordant patterns of amphotericin B activity *in vitro* and *in vivo*. Specifically, the limited aqueous solubility of the drug at concentrations at or near the MIC coupled with the unusual high degree of concentration-dependent protein binding may work in concert to create a ‘pharmacodynamic ceiling’ for the drug that cannot be overcome with higher dosages.\(^13\) To examine this possibility, we examined the influence of HSA on the concentration-dependent fungicidal activity of amphotericin B and found a significant decrease in amphotericin B activity that, in the case of a *C. lusitaniae* isolate with an MIC of 1.5 mg/L, resulted in complete loss of fungicidal activity even at drug exposures in excess of \(10\times\) MIC. When a bolus and continuous infusion strategy were compared in the setting of physiological concentrations of albumin, no significant differences in antifungal activity were observed for the two regimens, even though exposures achieved with the bolus regimen resulted in an AUC\(_{0–t}\) that nearly doubled the concentrations that were achieved with the continuous infusion regimen. We believe that in the presence of protein, the maximal biologically active concentration of amphotericin B available for killing of *Candida* in the system is <1000 ng/mL. Because both regimens achieve this threshold concentration, it is not surprising that the bolus regimen provided no discernable improvement in fungicidal activity over the continuous infusion.

Other investigators have reported substantial decreases in the activity of amphotericin B against *Candida* species when the drug is tested in the presence of plasma proteins. Zhanel *et al.*\(^7\) reported no change in amphotericin B MICs for *C. albicans* when human serum was added to wells during broth microdilution susceptibility testing. However, in time–kill studies, the addition of human serum reduced the fungicidal activity of amphotericin B by over \(3 \log_{10}\) cfu/mL.\(^21\) *In vivo*, most animals studies evaluating the pharmacodynamics of amphotericin B have also reported a plateau effect once serum levels approach \(\sim 1\) mg/L. In a dosage fractionization study of amphotericin B pharmacodynamics using a murine model of invasive candidiasis, Andes *et al.*\(^7\) found that peak serum levels were the best predictor of amphotericin B activity *in vivo* but the effect began to maximize at a peak:MIC of \(\sim 4\) or corresponding serum levels of roughly 1 mg/L. However, additional drug activity was observed even up to a serum level of 2 mg/L and \(C_{\text{max}}\):MIC of 10. Fungicidal activity at this point in the dose–response curve, however, was only observed with a single datum point. Using a similar study design, we have recently completed a pharmacodynamic analysis of amphotericin B in the treatment of acute invasive aspergillosis in neutropenic and corticosteroid-immunocompromised mice.\(^22\) Maximal reductions in *Aspergillus* lung fungal burden (as determined by real-time quantitative PCR) were observed with dosing regimens that optimized peak drug levels in the plasma but plateaued once plasma drug concentrations reached 1 mg/L.\(^22\)

The solubility threshold and unbound fraction of bioactive amphotericin B probably differs among different tissue sites. Collette *et al.*\(^23\) examined the tissue concentrations, bioactivity and tissue fungicidal/fungistatic titres of organ specimens recovered from patients with cancer who had received amphotericin B deoxycholate therapy. Although high concentrations of amphotericin B were measured by high-performance liquid chromatography (HPLC) in the liver, spleen and lung (mean concentration, 27.5, 5.2 and 3.2 mg/mL, respectively), amphotericin B concentrations measured by bioassay were, on average, \(<20\%\) of concurrent concentrations measured by HPLC. None of the organ homogenates demonstrated fungicidal activity against *C. albicans* or *Aspergillus fumigatus*. In a similar study, Christiansen *et al.* documented high amphotericin B concentrations in the liver, spleen and lungs of patients with cancer who had received treatment with amphotericin B deoxycholate.\(^24\) Viable *Candida* and *Aspergillus* isolates (MIC < 0.4 mg/L) could be recovered, despite organ-tissue concentrations of 2.5–166 mg of amphotericin B per gram of tissue.\(^24\) An unanswered question is whether similar degrees of protein binding occur in anatomically restricted sites where amphotericin B penetration is limited (e.g. the brain, the heart and the vitreous humour). Dosage escalation may be more relevant for infections in these sites where drug concentrations are less saturated compared with, for example, the liver, where concentrations probably exceed solubility/protein binding thresholds by 1000-fold.\(^24\)

Collectively, our results contribute to a growing body of *in vitro* and *in vivo* data demonstrating the importance of drug solubility and protein binding on the pharmacodynamic activity of amphotericin B. These biopharmaceutical properties could have important implications for the performance and interpretation of susceptibility testing for amphotericin B. Moreover, limited solubility and high protein binding could be important factors that limit the efficacy of escalating amphotericin B dosages. A pharmacodynamic ceiling could explain, for example, why lipid formulations of amphotericin B that achieve 10- to 50-fold higher concentrations of total drug in the plasma still have roughly equivalent efficacy as the conventional amphotericin B formulation. Although our results provide no reason to believe that continuous infusions would be less effective than rapid infusions of amphotericin B in the treatment of *Candida* infections, extrapolation of our results to the *in vivo* efficacy of these regimens in humans would be purely speculation. Prospective randomized trials are required to confirm the effectiveness of this novel administration technique in the treatment of proven invasive mycoses.
Pharmacodynamics of rapid versus continuous AMB infusions

Acknowledgements

Presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 2003. Abstract M-1259. This study was supported by funding from the principal investigator (R.E.L.).

Transparency declarations

R.E.L.: Speaker’s Bureau (Merck & Co, Inc., Astellas, Schering-Plough); Research Support (Astellas, Enzon); Advisory Board (Astellas, Schering-Plough). R.A.P.: Research Support (Enzon); Advisory Board (Enzon). D.P.K.: Speaker’s Bureau (Merck & Co, Inc., Astellas, Schering-Plough); Research Support (Astellas); Advisory Board (Schering-Plough).

References