High purity amphotericin B

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Objectives: Amphotericin B (AmB) is a drug of choice for treatment of disseminated fungal infections, but its use is often associated with severe adverse effects. Our observation that generic formulations of AmB contain multiple polyene components led us to propose that removal of other polyenes would yield a high purity AmB (AmBHP) with an improved therapeutic index.

Methods: To test that premise, AmBHP was first isolated from generic AmB by semi-preparative reverse phase high-pressure liquid chromatography and then its effects were compared in vitro and in vivo with those of commercial AmB formulations.

Results: AmBHP proved to be as active as generic AmB against Candida albicans in vitro and as efficacious as both generic and lipid-complexed AmB in a Candida-infected mouse model. AmBHP appeared to be less toxic to human THP-1 monocytic cells than was generic AmB at low concentrations (<2 μM), as indicated by exclusion of Trypan Blue and incorporation of [3H]thymidine. At higher concentrations, effects of AmBHP and generic AmB (Pharma-Tek AmB, PTAmB) on thymidine incorporation and cytosolic calcium concentration were similar. General toxicity to AmBHP in vivo, as indicated by its apparent LD50 and survival of Candida-infected mice, was roughly twofold less than that to generic or lipid-complexed AmB. Likewise, AmBHP decreased mean glomerular filtration rate about half as much as did a 10-fold lower dose of PTAmB.

Conclusions: Taken together, these data indicate that AmBHP may represent a refinement of currently marketed AmB formulations, offering equal, if not better, efficacy with less toxicity.

Keywords: mycology, antifungals, polyenes

Introduction

The current armamentarium against disseminated fungal infections includes only four drug classes: polyenes [amphotericin B (AmB)], azoles (ketoconazole, fluconazole, itraconazole and voriconazole), echinocandins (caspofungin, anidulafungin and micafungin) and allylamines (terbinafine). Currently, a polyene is one drug of choice for serious, disseminated fungal infections and is often selected because of its broad spectrum of activity and fungicidal action. Yet, the usefulness of polyenes is often limited by significant toxicity. These considerations, together with the predicted increased incidence of disseminated fungal infections, provide a strong impetus for the development of new antifungal therapies or improvement of existing ones. In regard to the latter approach, multiple polyenes are produced by Streptomyces nodules and could be useful as lead compounds. We observed antymycotic activity by many of these polyene compounds, but their cellular toxicity appeared high relative to AmB. Thus, we hypothesized that purified AmB without the contaminating polyenes and perhaps other non-polyene contaminants present in commercial AmB formulations would attenuate adverse effects of the drug.1

Our purpose in this communication is twofold. One is to describe a semi-preparative high-pressure liquid chromatography (HPLC) procedure for the isolation of AmB and other polyene components from generic AmB, a commercial deoxycholate-based formulation. Generic AmB is readily available, affordable and representative of previous brand name deoxycholate-based formulations in its profile and the relative amounts of polyene ‘contaminants’. The second is to compare the effects of commercial formulations and HPLC-purified AmB, designated as high purity AmB or AmBHP, assessed in vitro and in vivo to challenge the premise that an AmBHP represents a refinement of currently marketed AmB by having a higher therapeutic index.

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Methods

AmB preparations

Generic AmB [Pharma-Tek AmB (PTAmB); Lot #788A8; Pharmatek Laboratories, Inc., San Diego, CA, USA] was obtained as a lyophilized powder containing AmB, sodium deoxylolate and sodium phosphate, respectively, in milligram ratios of 50:41.20. Other AmB preparations formulated with deoxylolate were obtained through local distributors including: Apothecon brand AmB (Lot# P8E313; Bristol-Myers Squibb, Princeton, NJ, USA), VHA brand AmB (Lot# 8A06892; repackaged Apothecon brand AmB), Sigma Chemical solubilized (Lot # 37H4001) and non-solubilized AmB (St Louis, MO, USA), United States Pharmacopea grade AmB (USP AmB; Lot# UMMC1; Alpharma ApS, Copenhagen S, Denmark) obtained as a non-solubilized formulation and lipid formulations of AmB (Lot# 1542, lipid complex; Enzon Pharmaceuticals, Bridgewater, NJ, USA; or liposomal; Astellas Pharmaceuticals, Deerfield, IL, USA). The Sigma Chemical brand agents were not manufactured or formulated for human use.

AmB high purity

Semi-preparative HPLC was performed by applying aliquots of generic AmB reconstituted in HPLC-grade water to a 10 mm (ID) × 250 mm 5 μm AquaC18™ column (Phenomenex USA, Torrance, CA, USA) and resolved with a gradient of methanol and 5 mM sodium phosphate (pH 5) at a flow rate of 1.5 mL/min. Elution was isocratic with 75% methanol/25% phosphate buffer for the first 44 min, followed sequentially by a linear gradient to 85% methanol/15% buffer over 6 min and then isocratic elution for another 40 min. All ratios are expressed as v/v. Components of interest were cleared of salt, after evaporation of the methanol, by concentration in a solid phase matrix (e.g. C18) and washing with 10 volumes of HPLC-grade water. Solutes were next eluted with methanol, methanol was evaporated and solutes were stored at 4°C. Immediately before use, solute (purified AmB) was dissolved in a minimal volume of dimethyl sulfoxide (DMSO) and then diluted with 15 mM phosphate buffer (pH 7.4) containing 4.1 mg/mL of sodium deoxylolate. The nominal purity of AmBHP was defined before isolation to be 95%. In practice, apparent purity of AmBHP varied between preparations from 96% to 99%. Estimates of purity are based on absorbance at 405 nm following resolution on a 4.5 mm (ID) × 133 mm C18 column during isocratic elution with 70% methanol/30% phosphate buffer (pH 5) at a flow rate of 0.5 mL/min. AmBHP was defined based on absorbance at 405 nm.

Spectrophotometric classification of PTAmB, AmBHP and other components resolved by HPLC was done on the basis of ultraviolet absorption. The ultraviolet spectra for tetraines [nystatin, amphotericin A (AmA)] have characteristic peaks near 290, 305 and 318 nm, whereas those for heptaines (AmB) occur near 360, 378 and 405 nm. Pentaines and hexaenes share peaks at other wavelengths, making it difficult to use other absorbance maxima to exclude them. Final polyene concentrations between samples were estimated on the basis of absorbance at 405 nm.

Susceptibility testing of yeast

Candida albicans (ATCC 44 858) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Stock solutions of each dehydrated AmB HPLC fraction (~60 s eluent intervals) were prepared by wetting powder in DMSO and then diluting with RPMI 1640 buffered to pH 7.0 with 0.165 M MOPS (PML Microbiologicals, Wilsonville, OR, USA). Effects of DMSO on MIC were tested using CLSI (formerly NCCLS) broth microdilution guidelines.2 An MIC90 was determined for all fractions and the reference amphotericin (PTAmB). A minimum fungicidal concentration (MFC) or minimum bactericidal concentration was determined for PTAmB and AmBHP. The MIC was defined as the lowest test concentration that allowed no detectable growth. The MFC was defined as the lowest test concentration that killed 100% of the organisms.

In vitro cellular toxicity

Assessment of AmBHP cellular toxicity was performed in a cultured cell system using human THP-1 monocytes (ATCC 212). Cells were routinely grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, streptomycin (100 mg/L) and penicillin (100 U/mL) at 37°C under a humidified atmosphere of 5% CO2 in air. Initial comparisons between PTAmB and AmBHP were made on cell viability, cytokine release and calcium signalling. Concentration–response relationships were established for PTAmB, USP-AmB and AmBHP over a range that included therapeutic concentrations (i.e. ≤5.4 μM).

Cell viability. The ability of THP-1 cells to exclude Trypan Blue, monitored by light microscopy using routine procedures, was used to provide an initial comparison of cytotoxicities to individual HPLC fractions relative to the cytotoxicity of PTAmB and Sigma AmB. Thymidine incorporation was assessed by incubating THP-1 cells growing in RPMI containing 10% fetal bovine serum, 25 mM HEPES with [3H]thymidine (1 μCi/106 cells; 62.5 pmol/μCi) (Perkin Elmer, Boston, MA, USA). Logarithmically dividing cells were collected by centrifugation and resuspended in RPMI containing 25 mM HEPES supplemented with 20% fetal bovine serum. Tritiated thymidine was added to the suspension, and cells were immediately aliquoted to replicate 48-well culture dishes (2 × 104 cells/well) for incubation in the absence (control) or presence (0.25–20 μM) of AmBHP, PTAmB or USP AmB. After 24 h incubation, 100 μL aliquots of each suspension were collected on glass fibre filters, the filters were washed and radioactivity on the filters was measured by liquid scintillation counting.

Cytokine release. THP-1 cells were resuspended to a final concentration of ~5 × 106 cells/mL, seeded in Limbro 24-well plates and incubated for 24 h at 37°C in 5% CO2. AmB formulations were then added to wells at final concentrations <20μM and then incubated for an additional 2 h. Supernatants were collected from each well after three freeze–thaw cycles and stored at ~−80°C until assayed. Samples were assayed for interleukin (IL)-1β using an enzyme-linked immunosorbent assay (Cistron Biotechnology, Pine Brook, NJ, USA). Manufacturer’s data indicate an assay sensitivity of 20.0 pg/mL and a specificity for IL-1β with no cross-reactivity for IL-1α, IL-2, tumour necrosis factor-α (TNF-α) or γ-interferon. Evaluations of assay precision demonstrated a coefficient of variation of 5.3% to 6.7% for intra-assay variability and 6.6% to 8.4% inter-assay variability.

Calcium signalling. Changes in cytosolic-free calcium concentration ([Ca2+]i) were measured to assess general mechanisms of intracellular signalling. Measurement of [Ca2+]i in THP-1 cells was based on the use of fluorescent dyes [acetoxymethyl ester of furaPE3 (2 μM) (Tef Labs, Austin, TX, USA)] described previously.3–5 Either PTAmB or AmBHP was added to the superfusate to a final concentration of 5.14 μM. Fluorescence measurements were corrected for autofluorescences of cells and drug prior to calculation of [Ca2+]i.
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**In vivo studies**

**Renal and haemodynamic assessment.** Rats (Harlan Sprague Dawley 224–249 g; Harlan Teklad, Madison, WI, USA) underwent procedures and experimental designs that were in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and approved by the University of Mississippi Medical Center’s Institutional Animal Care and Use Committee. Animals were anaesthetized and cannulated in the left femoral artery and right femoral vein for blood sampling and blood pressure monitoring. All animals were instrumented with a specially made Silastic catheter and steel bladder as described by Gellai and Valtin for renal clearance measurements. After complete instrumentation, all animals were allowed to recover for a period of 4 days.

Pilot studies were performed to determine a minimum ‘nephrotoxic dose’ for PTAmB defined as a 15% to 20% reduction in renal function within 2 h. Doses studied for assessment of renal toxicity started as a single 1 mg/kg dose infused at 0.015 mL/min over 1 h. Incremental increases in dose were performed until a 16% mean reduction in mean glomerular filtration rate (GFR) was achieved with an AmB 2 mg/kg dose. A dose for AmBHP and lipid AmB agents was arbitrarily selected at 10-fold this dose. In experimental studies, animals were treated with saline for up to 1 day and then placed in modified restraining cages for renal function measurements. Each animal served as its own control with baseline renal function assessed prior to receiving AmB formulations. These animals were then crossed over into the treatment arm to receive PTAmB 2 mg/kg/h compared with AmBHP, AmB lipid complex (AmBLC) or liposomal AmB at 20 mg/kg/h. After drug or saline administration, the femoral vein catheter was infused with isotonic saline containing sodium [123I]iothalamate and para-aminohippurate (PAH) at a fixed rate of 3 mL/h. After a 60 min stabilization period, two 20 min urine collections were obtained, followed by collection of blood samples. Urine volume was determined gravimetrically. Sodium and potassium concentrations in plasma and urine were measured by flame photometry. GFR and effective renal plasma flow were calculated from the radioactivity of [123I]iothalamate and concentration of PAH, respectively, in plasma and urine. PAH concentration was determined colorimetrically. Arterial pressure was monitored in conscious animals with a pressure transducer (Cobe III Transducer CDX Sema, Birmingham, AL, USA) connected to a Grass model 7B chart recorder for continuous recording.

**Survival in an infection model.** Evaluation of efficacy was performed in a specific pathogen-free female BALB/c mouse (age 12–20 weeks: IFFA Credo; L’Arbresle, France), *C. albicans* ATCC 44 858 systemic infection model. Initial studies were performed to determine the infectious inoculum that caused 90% mortality 5 days post-infection, i.e. the 90% lethal dose (LD90). These inocula (3–5 × 10^6 cfu/mL) were used in all subsequent studies. A second pilot study also was performed to identify a non-toxic dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push to fasting dose of AmB. Increasing doses (1–15 mg/kg) of PTAmB were administered via the lateral tail vein by intravenous push.

On the basis of the above pilot studies, mice were infected with 0.15–0.2 mL of *C. albicans* inoculum (4 × 10^8 cfu/mL) by lateral tail vein injection and then returned to their home cages. Post-infection viability counts were performed to ensure that the correct inoculum had been given. Treatment began after 16 h with a starting dose of 0.5 mg/kg of a non-lipid AmB formulation or 5 mg/kg of a lipid formulation. At the time of death or animal sacrifice (5 days maximum), vital organs (liver, spleen, heart, bladder, muscle) were isolated, homogenized, diluted and plated on Sabouraud dextrose agar. Incubating plates were examined daily for 7 days, and colony counts were recorded from all plates each day. A second batch of organ tissue was evaluated microscopically. Leucopenia was not induced by administration of cyclophosphamide due to the overall success of the model and the desire to maintain competency of the immune system.

**Statistical analysis**

Animal survival between treated and untreated infected animals was selected as the primary outcome measure. These data were analysed using log-rank analysis. All other data are presented solely as demonstrative characterizations. First-order exponential decay was used to determine effective concentrations (ECs) during thymidine inhibition studies.

**Results**

Analytical HPLC demonstrated the presence of multiple components in a number of commercially available AmB formulations (Figure 1a). Although the chromatographs are qualitatively similar, there are considerable quantitative differences in the amounts of individual components. In fact, the amount of AmB, as indicated by absorbance at 405 nm, accounted for only 50% (VHA AmB) to 90% (Apothecon AmB) of the material present in the formulations. Sodium deoxycholate, which is added as a surfactant to non-lipid commercial formulations, had no detectable absorbance at 405 nm and made no contribution to the estimate of relative purity. UV/VIS absorbance spectroscopy of fractions corresponding to the polycene, AmB (i.e. AmBHP) as well as fractions corresponding to some earlier- and later-eluting peaks resolved by semi-preparative HPLC indicate that most of the components absorbing at 405 nm are polycene in nature (Figure 1c). All polycenes exhibited characteristic absorbance maxima near 365, 380 and 405 nm. A quantitative comparison of AmA, AmB and AmBHP was previously completed by absorbance spectrophotometry and reported. Nystatin was used as the tetraene control, representing AmA, and Apothecon brand AmB was the standard for the heptaene. There were distinct peaks at wavelengths of 345, 363 and 386 that correspond to either a pentaene or a hexaene in all AmB preparations. Pentaene (unique absorbance peak = 333 nm) quantities were highest in Sigma AmB (10.8%), and negligible (<6.7%) in the other AmB formulations. The maximum absorbance at 405 nm, together with additional absorbance peaks at 385, 363 and 345 nm, is consistent with AmBHP being a heptaene. AmBHP used in the studies reported here had a purity of 95% or greater (Figure 1b). That level of purity represents improvement over currently available non-lipid AmB formulations such as PTAmB (apparent purity of ~89%) and possibly the AmB used for lipid formulation.

Initial studies were performed to test the relative properties of the various polycene components in PTAmB that were resolved by analytical reverse-phase HPLC. In these experiments, column eluate was collected in 1 min fractions, with no attempt to pool...
Evidence for multiple polyenes in commercial deoxycholate-based AmB formulations. (a) Sigma chemical grade AmB (Sigma), Apothecon brand AmB (Apothecon), repackaged Apothecon brand AmB (VHA) and Pharma-Tek brand AmB (Pharma-Tek) were subjected to reverse-phase HPLC, and absorbance of column eluates was monitored at 405 nm. The peak eluting between 7 and 8 min was collected and designated as AmBHP. (b) Aliquots of Pharma-Tek AmB and two separate preparations of AmBHP were subjected to HPLC, and absorbance of the eluates was monitored at 405 nm. Apparent purities are indicated in parentheses. (c) Fractions corresponding to AmB (i.e. AmBHP) as well as some unknown compounds [designated by peaks 1, 2 and 3 in (a)] were collected during semi-preparative HPLC of an aliquot of Pharma-Tek AmB, and the absorbance spectrum of each fraction was subsequently measured. In (c), absorbance is expressed in arbitrary units. In (a) and (b), all chromatographs depicted in the respective panel are presented on the same time and absorbance scales, but baselines have been off-set to allow distinction of individual samples.
fractions corresponding to individual peaks in the chromatogram. AmBHP eluted as a single peak between 7 and 8 min, corresponding to fraction 8. Heptaene content of each fraction was estimated by absorbance at 405 nm. Samples were run in duplicate and at multiple concentrations when possible. MICs indicate that fractions corresponding to AmBHP as well as fractions eluting immediately before and after AmB had activity against the ATCC strain of *C. albicans* tested. The greatest activity was exhibited by fraction 8, corresponding to AmBHP, which had an MIC of 0.25 μM and an MFC of 0.5 μM. Fractions 6 and 7, which eluted immediately before AmBHP, each had an MIC of 1 μM, whereas the MFC for fractions 6 and 7, respectively, was 2 and 1 μM. Fractions containing earlier eluting polynes were not tested for MFC, but each had an MIC >1 μM. The MIC of AmBHP was >1 μM for fractions 2 and 4, and it was >2 μM for the remaining fractions. Finally, fraction 10, which eluted after AmBHP, exhibited an MIC of 0.5 and an MFC, respectively, of 0.5 and 2 μM. Clearly, fractions containing AmBHP had superior inhibitory and cidal activities compared with fractions containing other polyene components. AmBHP activity was compared with the commercial AmB formulation in triplicate experiments. Susceptibilities were identical for both formulations.

Toxicity of the polyene fractions was evaluated initially on the ability of THP-1 cells to exclude Trypan Blue. AmBHP dissolved in DMSO (<6%, v/v) or sodium deoxycholate did not affect cell viability at concentrations ≤10 μM. Concentrations of commercial AmB (AmBPT or USP AmB) >5.4 μM or Sigma AmB >3.5 μM are well recognized to induce cellular toxicity. Thus, on the basis of Trypan Blue exclusion, AmBHP appears to be less disruptive to the cell membrane than is commercial deoxycholate-formulated AmB. To refine our understanding of the relative cellular toxicity of AmBHP, a comparison was made between this and some other AmB formulations using incorporation of [3H]thymidine as an index of cytotoxicity. Incorporation of [3H]thymidine differentiates cell death from loss of viability since it measures the synthesis of new DNA. All three AmB preparations tested caused concentration-dependent reductions in [3H]thymidine incorporation. Inhibition by AmBHP or PTAmB was less pronounced than was inhibition by USP AmB, whereas the effects of AmBHP and PTAmB were largely indistinguishable (Figure 2). Sigma brand AmB was not used in thymidine assessments due to its overall toxicity observed with Trypan Blue. EC50 values calculated for these agents revealed an EC50 of 4.5 μM (PTAmB), 5.1 μM (AmBHP) and 1.3 μM (USP AmB) and an EC50 of 15 μM (PTAmB), 13.5 μM (AmBHP) and 4.3 μM (USP AmB).

The potential for AmB formulations to induce inflammation-related reactions can be assessed in vitro by their ability to promote the release of cytokines such as IL-1β. Control THP-1 cells incubated in the absence of AmB expressed no more than 120 pg/mL (7.1 fmol/106 cells/24 h) of IL-1β, whereas the expression of IL-1β increased to 300 pg/mL (17.6 fmol/106 cells/24 h) and 750 pg/mL (44.1 fmol/106 cells/24 h), respectively, in response to 0.11 and 10.8 μM PTAmB (Figure 3). Chemical grade AmB (Sigma AmB) caused substantially greater expression of IL-1β at concentrations >1 μM. In contrast, AmBHP caused less of a cytokine response than did either of the other AmB formulations. Although AmBHP increased IL-1β expression to rates comparable to those achieved in response to PTAmB and Sigma AmB at concentrations <1 μM, AmBHP failed to further increase cytokine release at concentrations as high as 21.6 μM. Cell counts at the end of each study demonstrated no differences in cell growth or mortality between groups (Figure 2).

We have demonstrated previously that the increase in IL-1β expression by THP-1 caused by PTAmB is accompanied by an increase in [Ca2+]i. Since the calcium signal in response to AmB might be as much an index of cytotoxicity as it is a reflection of intracellular signalling for pro-inflammatory cytokine release, studies were performed to compare the changes in [Ca2+]i caused by PTAmB with those caused by AmBHP. Not surprisingly, the data indicate that an increase in [Ca2+]i likely contributes to the effects of both PTAmB and AmBHP on cell function. It is also noteworthy that the calcium signals elicited by the two AmB preparations were similar in magnitude and kinetics. In light of the difference in apparent purities of the particular AmBHP (98%) and PTAmB (88%) preparations used in this experimental set, the similarities in the calcium signals suggest that an increase in [Ca2+]i may be a common action of all the polyene components. USP AmB elicited a calcium signal that was comparable to those caused by AmBHP and PTAmB (data not shown).

Renal toxicity associated with AmB formulations varied between agents. The AmB dose of generic (Pharma-Tek®) AmB was initially titrated to identify one that was associated with overall acute renal toxicity in vivo, yet not be associated with acute lethality. The final dose selected was 2 mg/kg. The doses for the AmB lipid formulations and for AmBHP were then arbitrarily chosen to be 10-fold greater, each administered for over 1 h. GFRs for control animals varied between 1.96 and 2.37 mL/min (mean = 2.19 mL/min). The mean GFRs of animals treated with AmBHP (2.03 mL/min), AmBLC (2.43 mL/min), liposomal AmB (1.83 mL/min) and AmB (1.92 mL/min) were difficult to compare with the mean GFR for control animals due to variance. However, when data are viewed as percent of their individual control values, one can easily notice the differences in renal function (GFR) of animals treated with AmBHP (7.9% decrease) or liposomal AmB (16.4% decrease) compared with the renal function of animals treated with PTgeneric AmB (12.3% decrease). The increase in renal function observed with AmBLC (10.9% increase) was also associated with increased sodium excretion and urine flow rate suggesting an increase in renal blood flow. It is also noteworthy that other indices of cardio-renal function were not adversely affected by AmBHP at doses well outside (10-fold) the range of toxicity for generic AmB in vivo. In fact, they compared favorably with values noted in animals treated with lipid formulations of AmB (Figure 4).

Efficacies of PT brand AmB (0.5 mg/kg), AmBHP (0.5 mg/kg) and AmBLC (5 mg/kg) were assessed after *C. albicans* inoculation by monitoring mortality for a maximum of 5 days post-infection. Initial studies (data obtained from five sets of mice) indicated that the LD50 for AmBHP (~5 mg/kg) is at least twice that of commercial AmB (~2.5 mg/kg) (data not shown). At 5 days post-infection, mortality from disseminated candidiasis in animals not treated with AmB was 90%. As illustrated in Figure 5, any treatment was significantly better than no treatment (P < 0.01). Even though survival between the three treatment groups was not statistically different, AmBHP appeared to have the greatest efficacy. Mortality in infected mice treated with AmBHP was only 20%, whereas mortality in
animals treated with either PTAmB or AmBLC was 40%. Thus, despite the lack of a statistically significant difference, a clear trend towards improved survival is observed with AmBHP in infected animals. That trend is potentially even more important in as much as AmBLC was given at a 10-fold greater dose. In any event, these data and data related to nephrotoxicity suggest that formulation of AmBHP with a lipid carrier would further improve both efficacy and safety in vivo.

The *C. albicans* tissue burden provided another assessment of relative efficacy. In general, results of post-mortem examinations of liver (1.7 ± 0.5 mean cfu × 1000 (mcfu) ± SEM), spleen (1.4 ± 1.3 mcfu), kidney (1.2 ± 0.8 mcfu), lung (22.3 ± 6.8 mcfu) and blood (0.1 ± 0.001 mcfu) indicate that the efficacy of AmBHP is comparable to those of PTAmB (76 ± 9, 6.9 ± 0.1, 0.7 ± 0.1, 3.3 ± 1.3, 0.01 ± 0.001) and AmBLC (3 ± 0.2, 1.6 ± 0.2, 0.2 ± 0.02, 39.8 ± 11.2, 0) for each organ, respectively. All colony counts for treatment groups were ~2 logs lower than the untreated controls. The highest colony counts were seen in the control animals lungs (751 mcfu) and kidneys (728 mcfu). Although no statistical analysis was planned or performed due to the small sample size, lower colony counts from lungs and livers of AmBHP-treated animals suggest that it might be more efficacious in those tissues than is generic AmB. Likewise, AmBLC appeared to have the greatest activity of the

Figure 2. Cell viability: effects of AmBHP, Pharma-Tek AmB and USP AmB on thymidine incorporation by THP-1 cells. Incorporation of \[^3H\]thymidine by THP-1 monocytic cells incubated in the absence (control, black diamonds) or presence of increasing concentrations (0.25–20 μM) of USP grade AmB (USP AmB, white diamonds), Pharma-Tek brand AmB (PTAmB, grey squares) or AmBHP (grey circles) was assayed over a 24 h period as described in the Methods section. Data are expressed as mean ± SEM (n = 3) of three separate experiments in which each condition was replicated twice. A first-order exponential decay curve was fitted to the mean data for each treatment group. The dashed horizontal line indicates one half the rate of thymidine incorporation by control (vehicle-treated) cells.
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![Graph](https://example.com/graph.png)

Figure 3. Cytokine release assay. This assay can predict infusion-related reactions associated with AmB formulation administration in man. THP-1 cells was (mononuclear like) exposed to varying concentrations of AmB formulations (black downward triangles, Sigma brand AmB; small white circles, Generic brand AmB; small black circles, AmBHP) release a pro-inflammatory cytokine which may be used as a marker for infusion-related toxicity. Data are expressed as mean ± SEM (n = 3) of three separate experiments in which each condition was replicated twice.

Discussion

The usefulness of AmB has always been compromised by a high incidence of adverse effects. Flu-like symptoms (fever, chills, myalgias), a capillary leak syndrome (hypotension, decreased organ perfusion), pulmonary congestion, changes in mental status (lethargy, confusion, agitation), renal dysfunction with secondary hypokalaemia, hypomagnesaemia and anaemia, and/or liver dysfunction are observed in up to 70% of patients treated with a generic formulation of AmB.\textsuperscript{9–11} Mechanisms for the flu-like syndrome include the expression of cytokines or prostaglandins by mononuclear cells that then alter the hypothalamic set point, inducing fever and chills. Exposure of mononuclear cells to AmB causes unique (ionophoretic) morphological changes associated with increased secretion of TNF-α and IL-1β.\textsuperscript{12,13} An increase in gene expression rather than secretion of preformed protein appears to be the prevailing mechanism by which AmB promotes cytokine release. AmB-induced activation of the IL-1β gene is mediated by calcium and due, in part, to the drug’s ionophoretic activity.\textsuperscript{13} Therefore, changes in cell membrane integrity and eicosanoid/cytokine production appear to be refined in vitro markers of AmB action/toxicity. In fact, clinical studies revealed that patients receiving AmB had higher IL-1β and TNF-α serum concentrations than did patients receiving azole antifungal agents and increases in cytokine concentrations correlated, at a minimum, with AmB infusion-related toxicities. In that regard, the fact that AmBHP caused substantially lower IL-1β production than did generic or chemical grade AmB strongly suggests that the use of AmBHP would be associated with fewer adverse reactions in vivo. That premise is supported, albeit indirectly, by at least a 2-fold increase in the LD₅₀ for AmBHP compared with PTAmB as well as a lower rate of mortality in mice infected with C. albicans after treatment with AmBHP than after treatment with generic AmB or AmBLC.

It is generally accepted that the initial action of AmB occurs at the level of the plasma membrane and results in the formation of pores of sufficient diameter to allow transmembrane movement of monovalent ions. This mechanism of action is independent of cell type and contributes to both the efficacy of AmB against fungi and its toxicity toward mammalian cells. Data presented in this communication and previously\textsuperscript{6} indicate that both generic PTAmB and AmBHP elicit a relatively rapid, sustained, but limited, increase in [Ca²⁺]. It is somewhat surprising that the calcium signal caused by AmBHP is indistinguishable from that caused by PTAmB since one might have predicted that removal of some of the ancillary components present in generic AmB would reduce or at least alter the calcium signal. It did not argue that an increase in [Ca²⁺] is a general response to all polyenes. It is likely that a rise in [Ca²⁺] is an obligatory consequence of the mechanism of AmB action and that a calcium signal per se is not a good predictor of cytotoxicity.

The cytotoxic effects of AmBHP were also evaluated by measurement of [³H]thymidine incorporation by THP-1 cells. Both PTAmB and AmBHP caused concentration-dependent reductions in thymidine incorporation. In general, the reductions in thymidine incorporation caused by AmBHP were not distinguishable from those caused by PTAmB. At face value, these data do not support the contention that low concentrations of AmBHP are less toxic. But it was not more toxic, even at concentrations as high as 20 μM. In fact, within the therapeutic range, inhibition of thymidine uptake occurred at a 2-fold lower concentration of generic AmB than of AmBHP. Given the general mechanism of action of AmB, the cytotoxic and non-cytotoxic effects of the drug most likely cannot be fully resolved. Even so, data from in vitro measurements of toxicity contrast significantly with data on the relative toxicity of AmBHP assessed in vivo. The positive effects of AmBHP on survival and renal function relative to other AmB formulations, coupled with equal or better efficacy, seem more relevant to its potential as a safer therapeutic agent for treatment of disseminated fungal infections. The explanation for the apparent difference in the toxicity of AmBHP in vitro and in vivo is not readily apparent. It is possible that differences in cytotoxicity between AmBHP and less pure preparations such as PTAmB would be apparent in a different cell type, e.g. a renal epithelial cell. Also, the kinetics of AmBHP and other polyene components present in currently available formulations of AmB might be different. Be that as it may, perhaps the most significant indication for the potential of AmBHP as a safer antifungal agent in vivo is the relatively modest increase in IL-1β production by mononuclear cells in response to it compared with other AmB preparations.

Drug-associated renal dysfunction is among the most clinically important AmB side effects. Researchers have estimated that up to 80% of patients receiving AmB will experience an episode of renal impairment during prolonged treatment,\textsuperscript{14} an additional 8.2 days of hospitalization along with a secondary 2–2.7-fold increased risk of death.\textsuperscript{15,16} The cost of AmB-induced events was $29,823 per case. A recent multivariate analysis of risks for AmB-related nephrotoxicity identified cumulative dose...
Figure 4. Nephrotoxicity. Rats were administered single doses of AmB 2 mg/kg, or AmBLC, liposomal AmB and AmBHP 20 mg/kg over 1 h. All animals entered into a match design, where saline was administered and renal function was assessed. After baseline was established, AmB formulations were administered in groups of five animals. These studies were performed in triplicate; however, statistical analysis was not performed due to sample size. The solid lines connect the means of the saline control (left) to means of treatment group (right).
High purity amphotericin B

Figure 5. Survival studies in disseminated candidiasis. Survival (log-rank) analysis was performed for animals (n = 5) receiving each AmB formulation or control saline in our murine candidaemia model. Each exposure is labelled: solid line, saline; short dashes, ABLC; dots, AmBHP; long dashes, PTAmB. Survival was superior in all three treatment groups compared with control (P < 0.01). None of the treatment groups was statistically different in outcome. AmBHP appeared associated with 80% survival at 120 h compared with 60% survival for PTAmB or ABLC.

of AmB and concomitant receipt of other nephrotoxic drugs (particularly, cyclosporine) as major predictive factors. In the studies reported herein, GFR and other indices of renal function in response to infusion of AmBHP were comparable to those caused by a 10-fold lower dose of generic AmB. Importantly, baseline values for GFR in our studies were within the standards reported by others using the same model. The use of lipid-based formulations of AmB, secondary to their lower risk for nephrotoxicity, is replacing conventional AmB therapy for treatment of systemic fungal infection except in many HIV-infected and paediatric patients. Yet, the cost of comparable therapy is considerable for the lipid formulation; daily cost for generic AmB averages $25, whereas that for lipid-formulated AmB ranges between $450 and $1850. Assuming a 14 day course of therapy, a patient will pay an average of $7000 more for a lipid-based, albeit safer, AmB product. The addition of AmBHP to a lipid formulation needs to be investigated.

Finally, assessment of efficacy was completed in a routine, murine, disseminated candidiasis model, and the data obtained allow for some important comparisons between AmBHP and currently available commercial AmB formulations. First, AmBHP caused less overall toxicity in vivo, as evident by a decrease in mortality, than does generic AmB. The LD50 in mice for AmB has been evaluated and published for multiple breeds. The average parenteral dose identified as the LD50 was 2.78 mg/kg (range 2.3–3.46), with no apparent difference between OF-1, Albino Webster-derived CD-1 and BALB/c mice. In the studies described here, all but one animal died within 24 h in response to this dose of generic AmB (n = 10). There was a difference in fed or fasting animals; the LD50 in fasting mice (1.51 mg/kg) was lower than the LD50 in fed animals (2.38 mg/kg). Formulation of AmB into an emulsion (LD50 7.34 mg/kg), a lipid complex (LD50 >75 mg/kg) or a liposome (LD50 32.9 mg/kg) significantly improves the LD50 when compared with AmB formulated with deoxycholate. Data obtained from five sets of mice indicate that the LD50 for AmBHP (>5 mg/kg) is at least twice that of commercial AmB (~2.5 mg/kg). Second, our data indicate that when doses are titrated within a non-lethal range, AmBHP appears as effective as other AmB formulations in controlling Candida infection. Moreover, reduction in tissue burden achieved with AmBHP appears to be associated with improved survival.

Toxicity of AmB appears to be impacted not only by the presence of impurities, but also by particle size. Particle size can impact parameters such as solubility, surface area, serum half-life and reactivity of the molecule. An in vivo study indicated that a deoxycholate-based colloid of AmB in which particle size was <0.3 μm was less toxic than were microcrystalline (0.3–0.5 μm) and crystalline (3–5 μm) suspensions of AmB. Additionally, the murine LD increased (3.4, 5 and 24.2 mg/kg, respectively) with increasing particle size. The deoxycholate-based colloid used by these investigators should be comparable to PTAmB used in the studies reported herein. Since AmBHP was initially dissolved in DMSO and then diluted in deoxycholate-phosphate buffer, its characteristics in solution are likely not directly analogous to those of PTAmB. AmBHP might remain ‘dissolved’ in micelles of DMSO, whereas PTAmB is present as a deoxycholate complex. In any event, as formulated in the current studies, AmBHP is not an aqueous suspension as are the crystalline preparations. Although the particle size of AmBHP has not been measured, there is a clear divergence between the relative efficacy and toxicity for AmBHP, which is in sharp contrast to the effects of AmB suspensions reported previously.

Conclusions
AmBHP may be a more effective, less toxic AmB formulation. When tested against CLSI strains of C. albicans, AmBHP is equally active in vitro, but more active in vivo when compared with other AmB formulations. Controlled trials will be required to assess clinical relevance of these in vitro and in vivo findings. In that regard, AmBHP could also be used as a lipid formulation to further improve the safety of these formulations.

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