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Development and characterization of oral lipid-based Amphotericin B formulations with enhanced drug solubility, stability and antifungal activity in rats infected with *Aspergillus fumigatus* or *Candida albicans*

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ABSTRACT

Objective: To develop an oral formulation of Amphotericin B (AmpB) with: (A) medium chain triglycerides, fatty acids and nonionic surfactants as a self-emulsifying drug delivery system (SEDDS); or (B) glyceryl mono-oleate (Peceol®) with poly(ethylene glycol) (PEG)-phospholipids.
Methods: SEDDS formulations were prepared by simple mixing at 40 °C. Peceol/DSPE-PEG-lipid formulations were prepared by solvent evaporation. Parameters evaluated included: miscibility, solubility and emulsion droplet size after incubation in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) via dynamic light scattering. The stability of AmpB in Peceol/DSPE-PEG was evaluated in SGF and SIF. Phase stability of AmpB in Peceol ± DSPE-PEG following thermal cycling was evaluated by atomic force microscopy (AFM). *Aspergillus fumigatus* ($2.9–3.45 \times 10^7$ colony forming units per mL [CFU]) or *Candida albicans* ($3–3.65 \times 10^6$ CFU per mL) were injected via the jugular vein; 48 h later male albino Sprague–Dawley rats (350–400 g) were administered either a single oral gavage of a Peceol®-DSPE/PEG2000-based AmpB (10 mg AmpB/kg and 5 mg AmpB/kg for the *Candida albicans* study only) twice daily for 2 consecutive days, a single intravenous (i.v.) dose of Abelcet® (5 mg AmpB/kg), or physiologic saline (non-treated controls; $n = 9$) once daily for 2 consecutive days. Antifungal activity was assessed by organ CFU concentrations and plasma galactomannan levels in the case of *A. fumigatus* and organ CFU concentrations in the case of *Candida albicans*. Plasma samples were taken from each animal prior to infection, 48 h after initiation of infection but prior to drug treatment and at the end of the study for plasma creatinine determinations as a measure of renal toxicity.
Results: Mean diameter of SEDDS after 30 min in 150 mM NaCl at 37 °C was 200–400 nm. However, the Peceol/DSPE-PEG, where PEG MW was 350, 550, 750 or 2000, showed a greater solubilization of AmpB (5 mg/mL) compared to SEDDS formulations (100–500 μg/mL). Upon dispersion in SIF, Peceol/DSPE-PEG formulations generated submicron emulsion particle sizes varying slightly with PEG MW. Stability of the AmpB in Peceol/DSPE-PEG formulations in SGF or SIF was >80% after 2 h, and best for formulations containing DSPE-PEG 750 or 2000 compared to 350, 550 or Peceol only. Monoglyceride-Peceol-DSPE/PEG2000-based oral AmpB treatment significantly decreased total fungal CFU concentrations recovered in all the organs added together by >80% compared to non-treated controls without significant changes in plasma creatinine levels in the *A. fumigatus* infected rats. In addition, this formulation significantly decreased kidney fungal CFU concentrations by >75% at the 5 mg/kg dose and by >95% at the 10 mg/kg dose compared to non-treated controls without significant changes in the plasma creatinine levels in the *Candida albicans*-infected rats.

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Conclusions: Novel lipid-based AmpB oral formulations were prepared that provide excellent drug solubilization, drug stability in simulated gastric and intestinal fluids and antifungal activity without renal toxicity in rats infected with *A. fumigatus* and *C. albicans*.

1. Introduction

In developed nations, disseminated fungal infections such as candidiasis, histoplasmosis and aspergillosis are on the rise, affecting patients with cancer, organ transplant recipients, diabetics and those with HIV/AIDS. In these patients, invasive fungal infections may account for as many as 30% of deaths. Despite the development of a number of new antifungal agents, Amphotericin B (AmpB; Fig. 1) formulated as an IV administered micelle and liposomal dispersion remains one of the most effective agents in the treatment of systemic fungal infections. In addition, a variety of parenteral formulation approaches have been studied for AmpB (Esposito et al., 2003; Forster et al., 1988; Lavasanifar et al., 2001; Rajagopalan et al., 1988; Tiyaboonchai et al., 2001; Yu et al., 1998). While effective, the limitations of these parenteral formulations of Amphotericin B are the safety issues associated with administration (infection of the indwelling catheter, patient chills and shaking due to RBC haemolysis, dose-dependent renal toxicity, etc.), feasibility of administration of parenteral products in remote locations and high drug cost.

The development of an effective and safe oral formulation of Amphotericin B would have significant applications in the treatment of disseminated fungal infections and would dramatically expand access to the treatment of visceral leishmaniasis. However, the bioavailability of AmpB is negligible due to low aqueous solubility, poor membrane permeability and instability at the low pH found in gastric fluid. Recently, attention has been focused on the use of oral lipid excipients to enhance the solubility and bioavailability of poorly water soluble lipophilic drugs (Gursoy and Benita, 2004). Oral lipid excipients consisting of: (A) medium chain triglycerides, fatty acids and nonionic surfactants designed to achieve self-nanoemulsification drug delivery systems (SEDDS) in aqueous media (Gershanik and Benita, 2000); or (B) glycerol monooleate (Peceol) with poly(ethylene glycol) (PEG)-phospholipids were explored as solubilizing agents for AmpB followed by the characterization of stability in simulated gastric fluid and simulated intestinal fluid.

Importantly, preliminary in vivo experiments exploring the potential for bioavailability enhancement of AmpB using a simple suspension in Peceol showed significant oral absorption and efficacy against systemic *Aspergillius* in a rat model (Risovic et al., 2007; Sachs-Barrable et al., 2008). The mechanisms by which Peceol enhances oral AmpB bioavailability are presently under study, but may involve combination of drug solubilization, enhancement of uptake into the lymphatic system and/or a specific effect on enterocyte permeability or drug transport pathways (Risovic et al., 2004; Sachs-Barrable et al., 2007). While very encouraging, the preliminary studies used a simple and uncharacterized suspension of AmpB in Peceol used immediately after preparation (Risovic et al., 2007). The current study endeavors to improve AmpB solubility and to determine the effect of simulated gastric and intestinal fluids on AmpB stability in lipidic formulations. The two approaches were SEDDS formulations with submicron emulsification properties and adding a surface-active agent to the AmpB/Peceol mixture, which was a phospholipid modified with a hydrophilic polymer, to enhance AmpB solubility in Peceol.

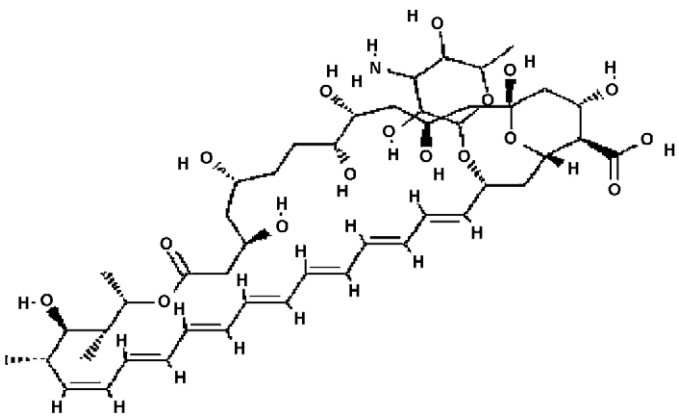

Fig. 1. Chemical structure of Amphotericin B (AmpB).

2. Materials and methods

2.1. Materials

Amphotericin B (from *Streptomyces* sp., Calbiochem, >86% purity) (Fig. 1) was purchased from EMD Biosciences (San Diego, CA) and used without further purification. Amphotericin B as the commercially available deoxycholate micelle dispersion (Fungizone®) was purchased from Vancouver General Hospital pharmacy. Phospholipids and poly(ethylene glycol)-lipids were all from Avanti Polar Lipids (Alabaster, AL). HPLC grade solvents were from Fluka. Peceol® (glyceryl oleate), Labrasol® (caprylocaproyl macrogol glycerides) and Gelucire 44/14® (lauroyl macrogol glycerides) were a gift from Gattefossé Canada (Mississauga, Ontario). Captex 355® and Capmul® were a gift from Abitech. Simulated gastric fluid (SGF) without enzymes was composed of 30 mM NaCl, titrated to pH 1.2 with 1N HCl. Simulated intestinal fluid with pancreatic enzymes (SIFe) was prepared according to the US Pharmacopeia method (USP28) as modified by Vertzoni et al. (2004), and was composed of 0.2 M NaOH, 6.8 g/L of monobasic potassium phosphate and 10 g/L of pancreatin (Sigma), adjusted to pH 7.5 with NaOH. Fasted-state simulated intestinal fluid with bile salts (FaSSIF) (Vertzoni et al., 2004) was composed of 3 mM sodium taurocholate (Sigma), 3.9 g/L sodium dihydrogen phosphate, 6.2 g/L NaCl in water, either with or without 0.75 mM lecithin and then titrated to pH 6.5 with NaOH. Water was purified by a reverse osmosis system and filtered (0.2 μm) prior to use. All other chemicals were of reagent grade purchased from Sigma–Aldrich.

2.2. Methods

2.2.1. Preparation of self-emulsifying drug delivery systems (SEDDS)

AmpB was mixed with the SEDDS lipid vehicles by combining the drug powder with the lipids followed by mild heating and stirring (45 °C for 1–2 h), protected from light. Any visible remaining drug particulates were removed by centrifugation at 10,000 × *g* for 15 min.

2.2.2. Preparation of Peceol/DSPE-PEG lipid formulations

AmpB was mixed with Peceol® and distearoylphosphatidylethanolamine (DSPE)-$(PEG)_n$ (where n = average PEG molecular weight, which varied as 350, 550, 750 or 2000). AmpB concentration was 5 mg/mL in the Peceol, to allow for complete drug solubilization in the initial mixture. The solution was stirred

for 1 h, protected from light, to dissolve AmpB and lipids, followed by solvent evaporation under vacuum (65 mbar) over several hours in a rotary evaporator. Ethanol was considered to be completely removed by achieving the original weight of the sample containing AmpB, Peceol and lipids measured immediately prior to the addition of the ethanol. A translucent yellow mixture without particulates was formed. No degradation or UV spectral shape changes of AmpB were observed following this processing.

2.2.3. Characterization of Amphotericin B stability in lipid formulations

Drug concentrations were measured by reverse-phase HPLC/UV or by UV spectrophotometry (λ = 407 nm). For HPLC analysis of AmpB, samples were diluted in 20% (v/v) methanol in DMSO and 20 μL were injected on a Luna 5 μm (2.0 mm × 150 mm) C18 column (Phenomenex) at 30 °C. The mobile phase was 10 mM sodium acetate and acetonitrile using a gradient program by the method of Wasan et al. (1990) on a Waters 996 HPLC system, detected by a Waters photodiode array detector (λ = 408 nm). Run time was 13 min and retention time was approximately 8.5 min. Stability of AmpB against decomposition or superaggregation (Sánchez-Brunete et al., 2004; Tancrède et al., 1990) upon mild heating of the lipid vehicles during drug solubilization and upon storage (21 °C) over 14 days was assessed by UV spectral shift analysis using a Thermoscan UV/visible spectrophotometer with λ = 250–500 nm. Nanoscale phase separation was observed by atomic force microscopy (AFM) at ambient temperature following one cycle of melting at 43 °C. AmpB formulated in Peceol alone or Peceol/DSPE-PEG2000 was briefly warmed to 43 °C and a thin film was manually applied to a single-side polished (1 0 0) crystalline silicon wafer coated with a 3-nm thick layer of silicon oxide (Silicon Sense, Nashua, NH). The silicon substrate was pretreated by immersing it for 15 min into a piranha solution, a 7:2 by volume mixture of concentrated sulfuric acid and 30% hydrogen peroxide, respectively. (*Caution*: This mixture reacts violently with organic material and should be handled with care.) Piranha solution removed organic residue from the surface of the silicon wafer. These substrates were subsequently rinsed with ~500 mL of 18.2 MΩ water and dried under a stream of nitrogen gas. Images were obtained with a MFP-3D-SA AFM (Asylum Research, Santa Barbara, CA) using silicon cantilevers (VistaProbes, VP30194) operating in tapping mode at 21 °C.

2.2.4. Stability in simulated gastric and intestinal fluids

Amphotericin B in Peceol/DSPE-PEG formulations (5 mg/mL) were prepared in triplicate and were incubated in SGF as a 1:10 (v/v) dilution or in SIF prepared with and without lecithin or with enzymes (as described above in Section 2.1) as a 1:50 (v/v) dilution at 37 °C with vigorous stirring. Incubation times were 0, 10, 30 or 120 min. At each time point, AmpB concentration was determined by spectrophotometry using triplicate measures of absorbance (407 nm) after complete solubilization in 95% ethanol to clarify the samples, thereby also diluting the samples to the linear range of the UV assay. Values were normalized to the baseline at 330 nm and concentrations were calculated based on an Amphotericin B standard curve prepared in each fluid type ($r^2 > 0.99$). The linearity of the standard curve and concentration range of standards prepared in Peceol/DSPE-PEG were not affected by the type of simulated gastrointestinal (GI) fluid or by incubation time, however, separate triplicate standard curves were prepared for each formulation containing the various MWs of DSPE-PEG (350, 550, 750 or 2000).

Particle size analysis by dynamic light scattering (ZetaPALS instrument, Brookhaven Laboratories, New York, operating at 650 nm) was used to assess nanoscale self-emulsification properties by determining emulsion droplet size and polydispersity of droplet size. In the case of AmpB SEDDS formulations, emulsion droplet size was measured in physiological saline (150 mM NaCl) following 30 min incubation at 37 °C. For Peceol/DSPE-PEG AmpB formulations, emulsion droplet size was measured at 37 °C every 10 min in preliminary experiments and it was found that the mean diameter came to equilibrium by 1 h and remained stable. Drug stability was measured after 2 h, therefore 2 h was the time point used for reported emulsion droplet size analysis from samples incubated in simulated intestinal fluids. Two data analysis modes are available, which calculate a weighted mean hydrodynamic diameter based on a lognormal distribution, and a multimodal distribution to identify subpopulations centered on two or more mean diameters. Both values are reported where a bimodal distribution was detected.

2.2.5. Animal studies

Male albino Sprague–Dawley rats (350–400 g) were purchased from Charles River Laboratories (Wilmington, MA). The rats were surgically implanted with a port (Access Technologies) and catheter with access to venous blood by a similar method used for rabbits. The rats were housed in an animal care facility with a 12 h light–dark cycle and controlled temperature and humidity. The rats were given *ad libitum* access water and standard rat chow (Purina Rat Chow) for the duration of the study. The ports were primed daily with normal saline and heparin to prevent blockages. The animals were cared for according to principals promulgated by the Canadian Council on Animal Care and the University of British Columbia.

2.2.6. Aspergillus fumigatus studies

A. fumigatus ($2.7–3.3 \times 10^7$ colony forming units [CFU]) was injected via the jugular vein; 48 h later male albino Sprague–Dawley rats (350–400 g) were administered either as a single oral gavage of monoglyceride (Peceol®)/DSPE-PEG2000-based AmpB (10 mg AmpB/kg; n = 7) twice daily for 2 consecutive days, a single intravenous (i.v.) dose of Abelcet® (5 mg AmpB/kg; n = 4), or physiologic saline (non-treated controls; n = 9) once daily for 2 consecutive days. Organs were harvested at sacrifice (day 3) and processed (see below). Blood was drawn before inoculation (blank), pre-dose (0 h) and 48 h after treatment for plasma creatinine analysis.

2.2.6.1. A. fumigatus inoculum. The *A. fumigatus* was a clinical isolate from a pool of patients with disseminated aspergillosis (BC Centre for Disease Control). Cultures were grown on sabouraud dextrose agar for 48 h at 37 °C. Conidia were isolated by washing the agar with pyrogen-free saline. The conidia were suspended by vortexing with glass beads and diluted with pyrogen free saline to obtain between 0.87 and 1.1×10^7 conidia in 300 μL of saline. Conidia were counted using a hemocytometer and a 100 μL aliquot was serially diluted and aliquots were plated on Sabouraud dextrose agar for 48 h at 37 °C to determine the number of viable conidia and purity of the inoculum. The average percentage of viable conidia in the inoculum was 62 ± 19. None of the spore suspensions were contaminated with any other organism. Rats were inoculated with 300 μL through the indwelling port 48 h before the beginning of treatment to allow for aspergillosis to develop.

One-milliliter whole blood samples were drawn into pediatric collection tubes (3.6 mg K_2 EDTA) before infection (blank), pre-dose (0 h) and 48 h after treatment (48 h). All whole blood samples were mixed by inversion and plasma was separated by centrifugation (15 min, 3000 rpm at 4 °C). Plasma samples were stored at −20 °C for creatinine analysis. After the collection of the 48 h blood specimen, the rat was euthanized with intravenous overdose (1 mL) of Euthanyl® (sodium pentobarbital 240 mg/mL). Spleen, right kidney, liver, lung, heart and brain tissue samples were harvested, weighed and placed in sterile containers. Normal saline was added, 1 mL/g of specimen and homogenized (Heidolph diax 900). An aliquot of organ homogenate was stored at room temperature until plating.

The choice of organ CFU as an indicator of antifungal activity was based on previously published work (Sivak et al., 2004). Aliquots of 100 μL full strength organ homogenate and 1:10 dilution (with sterile saline) were each spread plated onto Saboraud Dextrose Agar plates in duplicate. After 48 h incubation at 37 °C, the resulting colonies of *A. fumigatus* were counted and averaged over the duplicate plates. The limit of detection of the assay was 0.1×10^2 CFU/mL homogenate.

Renal toxicity was indirectly assessed, as previously described (Sivak et al., 2004), by determining creatinine concentration in plasma using a commercially available kit (Thermo Fisher Scientific Inc. Middletown, VA, USA). A baseline was determined by measuring creatinine concentration in the blank sample, and was compared to plasma creatinine concentration in the 0 h (pre-dose), 48 h samples. For the purposes of this study, a 50% or greater increase in plasma creatinine concentration as compared to baseline was considered to be a sign of renal toxicity.

2.2.7. Candida albicans studies

C. albicans ($1–1.35 \times 10^6$ CFU) was injected via the jugular vein; 48 h later male albino Sprague–Dawley rats (350–400 g) were administered either as a single oral gavage of Peceol®/DSPE-PEG2000-based AmpB (5 mg AmpB/kg; $n = 5$ or 10 mg AmpB/kg; $n = 7$) twice daily for 2 consecutive days, a single intravenous (i.v.) dose of Abelcet® (5 mg AmpB/kg; $n = 3$), or physiologic saline (non-treated controls; $n = 9$) once daily for 2 consecutive days. Organs were harvested at sacrifice (day 3) and processed (see below). Blood was drawn before inoculation (blank), pre-dose (0 h) and 48 h after treatment for plasma creatinine analysis.

2.2.7.1. C. albicans inoculum. The *C. albicans* was a clinical isolate from a pool of patients with disseminated candidiasis (BC Centre for Disease Control). Cultures were grown on sabouraud dextrose agar for 48 h at 37 °C. Cells were suspended by vortexing a single colony in pyrogen free saline with glass beads and diluted with pyrogen-free saline to obtain between 0.9 and 1.0×10^6 conidia in 300 μL of saline. Cells were counted using a hemocytometer and a 100 μL aliquot was serially diluted and aliquots were plated on Sabouraud dextrose agar for 48 h at 37 °C to determine the number of viable cells and purity of the inoculum. The average percentage of viable cells in the inoculum was 68% ±19. None of the cell suspensions were contaminated with any other organism. Rats were inoculated with 300 μL through the indwelling port 48 h before the beginning of treatment to allow for candidiasis to develop.

One-milliliter whole blood samples were drawn into pediatric collection tubes (3.6 mg K_2 EDTA) before infection (blank), pre-dose (0 h) and 48 h after treatment (48 h). All whole blood samples were mixed by inversion and plasma was separated by centrifugation (15 min, 3000 rpm at 4 °C). Plasma samples were stored at −20 °C for creatinine analysis. After the collection of the 48 h blood specimen, the rat was euthanized with intravenous overdose (1 mL) of Euthanyl® (sodium pentobarbital 240 mg/mL). Spleen, right kidney, liver, lung, heart, brain tissue and whole blood samples were harvested, weighed and placed in sterile containers. Normal saline was added, 1 mL/g of specimen and homogenized (Heidolph diax 900). An aliquot of organ homogenate was stored at room temperature until plating and the remaining sample was placed at −80 °C until HPLC analysis.

Aliquots of 100 μL full strength organ homogenate and 1:10 dilution (with sterile saline) were each spread plated onto Saboraud Dextrose Agar plates in duplicate. Whole blood samples were diluted to 1:100 final concentrations. After 48 h incubation at 37 °C, the resulting colonies of *C. albicans* were counted and averaged over the duplicate plates. The limit of detection of the assay was 0.1×10^2 CFU/mL homogenate. Renal toxicity was assessed in the same way as in *A. fumigatus* study.

2.3. Statistical analysis

The number of CFU's in organs and plasma creatinine concentrations prior to and following the administration of treatment were compared between each treatment group by analysis of variance (INSTAT2; GraphPad Inc.). Critical differences were assessed by Tukey post hoc tests. Serum creatinine values were compared prior to 48 h following treatment using repeat measures ANOVA with a Tukey post hoc test to determine critical differences (Prism 4; Graphpad Inc.). A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). All data related to the in vivo studies were expressed as a mean ± standard error of the mean.

3. Results

3.1. SEDDS formulations: solubility, physical stability and self-emulsification

SEDDS formulations had AmpB solubilities ranging from 100 to 500 μg/mL as measured by HPLC, compared to negligible solubility in aqueous solution (pH 7). As shown in Tables 1 and 2, mean particle diameter following 1:1000 (v/v) dilution in SGF at 37 °C was 200–400 nm at equilibrium for Captex 355/Capmul MCM/Tween 80 (Table 1) and Peceol/Gelucire 44/14 (Table 2) but not for formulations based on soybean oil, Peceol/Labrasol or Peceol/Gelucire 55/13 (>1 μm, data not shown). Visual observations were made regarding miscibility, phase separation and precipitation over several days at ambient temperature (21 °C). Several SEDDS formulations remained transparent and homogeneous, as indicated in Tables 1 and 2. For example, the Captex-based SEDDS formulations generated semi-transparent mixtures after mixing with water that were homogeneous for all combination ratios of Captex, Tween 80 and sodium phosphate (Table 1). Combinations of Peceol and Gelucire 44/14 in the range of 70/30 to 30/70 (v/v) generated a fine emulsion whereas some instabilities were observed when using Peceol with Gelucire 55/13 (Table 2). Submicron emulsions were achieved upon mixing with water for all of the Captex-based and Peceol/Gelucire SEDDS formulations of Amphotericin B.

3.2. AmpB solubility in Peceol/DSPE-PEG formulations and emulsification in fasted-state SIF

The combination of Peceol and DSPE-PEG_n, where average MW of PEG was varied from 350 to 2000, showed an even greater solubilization of AmpB (5 mg/mL) compared to the preliminary SEDDS formulations. At concentrations ≥10 mg/mL, some precipitation of AmpB did occur upon standing at ambient temperature (21 °C) over 24 h. Upon dispersion in SIF at 37 °C at 0.5 mg/mL followed by stirring for 30 min, the Peceol/DSPE-PEG2000 AmpB formulations generated translucent emulsions with particle sizes of 300–500 nm (Table 3) and no visible precipitate. In some cases, there appeared to be two populations of submicron particles, with some at 100 nm and others several hundred nanometer in diameter. It is unknown whether AmpB is preferentially associated with either subpopulation, which will require further study.

3.3. AmpB stability in simulated gastric and intestinal fluids

The chemical stability and aggregation state (monomeric vs. self-associated) of AmpB was evaluated in USP simulated gastric

Table 1
Captex 355-based Amphotericin B preliminary SEDDS formulations.

Sample ID	Components (%, v/v)				Visual observations after hydration	Particle size (nm)	
Sample	Captex 355	Tween 80	Capmul MCM	NaH_2PO_4 (10 mM, pH 4.0)		Supernatant	Mixed samples[a]
1	58	10	30	2	Semi-transparent	251.2	266.6
2	73	5	20	2	Semi-transparent	286.4	259.7
3	63	5	30	2	Semi-transparent	263.1	282.7
4	53	5	40	2	Semi-transparent	257.8	263.8
5	70	10	18	2	Semi-transparent	203.7	
6	50	17	31	2	Semi-transparent	176.4	274.9

For these mixtures, 3 mg of AmpB powder were combined with 0.3 mL (10 mg/mL) of the designated lipid combination and the mixture was stirred in a 1 mL amber glass vial at 37 °C for 2 h.
[a] Particle sizing was performed after mixture was dispersed 1:1000 (v/v) in 150 mM NaCl at 37 °C × 30 min and reflects mean diameter.

Table 2
Peceol/Gelucire Amphotericin B SEDDS.

Sample ID	Components (%, v/v)		Visual observations after hydration	Particle size (nm)[a]
System 1	Peceol	Gellucire 44/14		
1	70	30	Fine emulsion	174.3
2	50	50	Fine emulsion	320.1
3	30	70	Fine emulsion	290.9
Sample ID	Components (%, v/v)		Visual observations after hydration	Particle size (nm)[a]
System 2	Peceol	Gellucire 50/13		
1	70	30	Small amount ppt[b]	179.7
2b	50	50	Some ppt.	251.9
3c	30	70	Significant amount ppt	572.0

Components were mixed at 45 °C (system 1) and 55 °C (system 2) for 2 h. These temperatures are the melting points for Gellucire 44/14 and Gellucire 50/13, respectively.
[a] Particle sizing was performed after mixture was dispersed 1:1000 (v/v) in 150 mM NaCl at 37 °C × 30 min and reflects mean diameter.
[b] Precipitate, visible solid matter.

fluid as well as fasted-state simulated intestinal fluid with and without bile salts and pancreatin. As described in Section 2.1, AmpB in Peceol alone or in Peceol/DSPE-PEG formulations (PEG MW = 350, 550, 750 or 2000) was prepared at 5 mg/mL and incubated in the simulated GI fluids for a total period of 2 h. At 30 min intervals, the AmpB concentration and UV spectra were evaluated. AmpB exhibits five main spectrophotometric peaks in the UV range. Peaks 4 and 5 have the greatest amplitude in monomeric AmpB, whereas there is a left shift when AmpB becomes self-associated. Fig. 2A shows typical UV spectra of AmpB in Peceol/DSPE-PEG2000 at various concentrations over the linear range of the UV assay, illustrating the predominance of monomeric AmpB throughout this concentration range. This pattern was maintained when PEG MW was varied from 350 to 750 (data not shown to avoid redundancy). The same spectral pattern was also observed following incubation in SGF, as well as resulting in nearly identical standard curves for the various AmpB/Peceol/DSPE-PEG$_n$ preparations, as shown in Fig. 2B.

Regarding chemical stability, the trend was to slightly less drug stability in formulations prepared with DSPE-PEG 350 or 550 compared to DSPE-PEG 750 or 2000. AmpB alone (e.g. neat powder) was not soluble in these media and therefore could not be used properly as a control at comparable concentrations due to the confounding factors of increased dissolution over time vs. degradation. AmpB in Peceol alone prepared otherwise the same way was included as a negative control for the stabilizing effect of DSPE-PEG in the formulations. AmpB/Peceol showed a trend to slightly less drug stability in SGF than formulations containing DSPE-PEG 350 or 2000 as shown in Fig. 3. Fig. 4 shows AmpB stability in simulated intestinal fluid containing bile salts either without lecithin (Fig. 4A) or with lecithin (Fig. 4B) is less for AmpB in Peceol alone or in Peceol/DSPE-PEG 350 compared to formulations using the higher PEG MWs.

Table 3
Peceol/DSPE-PEG Amphotericin B formulations.

Formulation: Peceol/DSPE-PEG*n*, *n* = (MW)	Effective diameter (nm) lognormal distribution	Polydispersity index	Bimodal distribution median diameter (nm)	Subpopulation diameter range (nm) (%, v/v)
350	370	0.344	I: 155 II: 1067	I: 129–186 (20%) II: 888–1282 (80%)
550	600	0.402	I: 136 II: 1702	I: 108–171 (20%) II: 1206–1909 (80%)
750	596	0.395	I: 168 II: 1392	I: 134–210 (18%) II: 1245–3400 (82%)
2000	533	0.392	I: 154 II: 1581	I: 119–200 (30%) II: 1390–2330 (70%)
AmpB in Peceol alone	351	0.333	I: 151 II: 802	I: 128–194 (20%) II: 738–1120 (80%)

Particle sizing by dynamic light scattering of AmpB in Peceol/DSPE-PEG, where the MW of PEG was varied from 350 to 2000, following 2 h incubation simulated fasted-state intestinal fluid (pH 6.8) at 0.5 mg AmpB/mL.

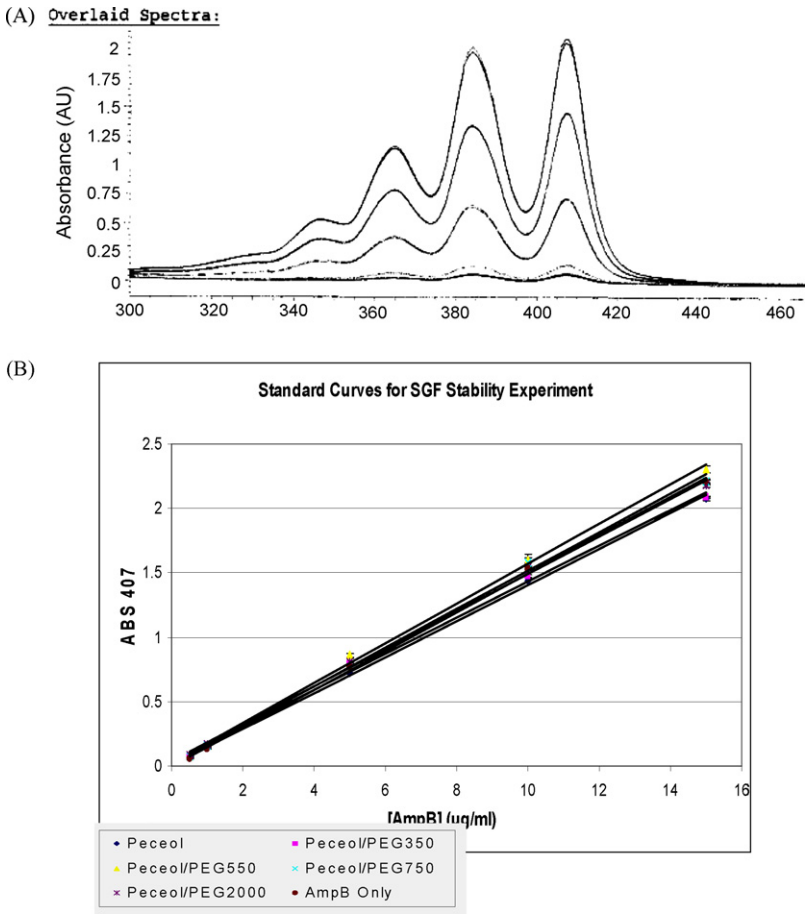

Fig. 2. (A) UV absorbance spectra over time of AmpB in Peceol/DSPE-PEG at various concentrations following incubation in simulated gastric fluid. There is no change in the peak height or peak ratio at any concentration as a function of incubation time up to 60 min. The curve series represents the UV absorbance vs. wavelength of increasing concentrations of AmpB. (B) Data from (A) were combined using peak height at 407 nm to construct the standard curves of AmpB absorbance vs. concentration. A different standard curve was prepared for each formulation where DSPE-PEG molecular weight varied.

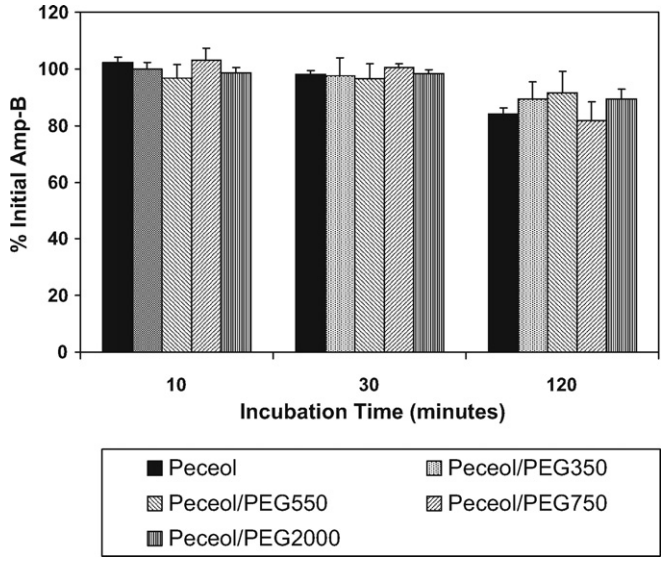

Fig. 3. Stability of AmpB in Peceol/DSPE-PEG at 37 °C in simulated gastric fluid. Data represent the mean ± S.D. of three independent experiments, each of which was performed in triplicate.

Varying the DSPE-PEG MW had no clear effect on the emulsion droplet size in simulated intestinal fluid (Table 3) following mixing over a period of 2 h at 37 °C. Submicron mean diameters were observed in the range of 300–600 nm with a fairly wide polydispersity. A bimodal particle size distribution was also generated, with a small subpopulation centered in submicron range (150–300 nm) and another centered in the 1–2 μm range. AmpB in Peceol alone also formed droplets of similar size and distribution in simulated intestinal fluid. These particle size measurements were performed in the absence of lecithin, which formed very large emulsion droplets under the mixing conditions employed and opacified the samples.

Fig. 5 illustrates the stability of AmpB in simulated intestinal fluid with pancreatin, which contains degradative enzymes. These data suggest better stability of formulations containing DSPE-PEG 750 or 2000 compared to 350 or 550 or in Peceol alone. In evaluating the degradation of AmpB in Peceol alone, however, it is important to note that poor mixing of AmpB/Peceol in the simulated GI fluids were observed. No changes associated with conversion of the monomeric form vs. aggregated AmpB, such as a difference in the height ratios of specific peaks in the UV spectra or overall pattern was observed following the full incubation time in the various media described here (data not shown).

Stability against phase separation of lipidic components and the presence of drug crystals on the nanoscale level were determined by imaging the samples by AFM techniques. AmpB in Peceol or Peceol/DSPE-PEG2000 was melted at 43 °C and applied to a hydrophilic substrate and imaged at 21 °C. Fig. 6

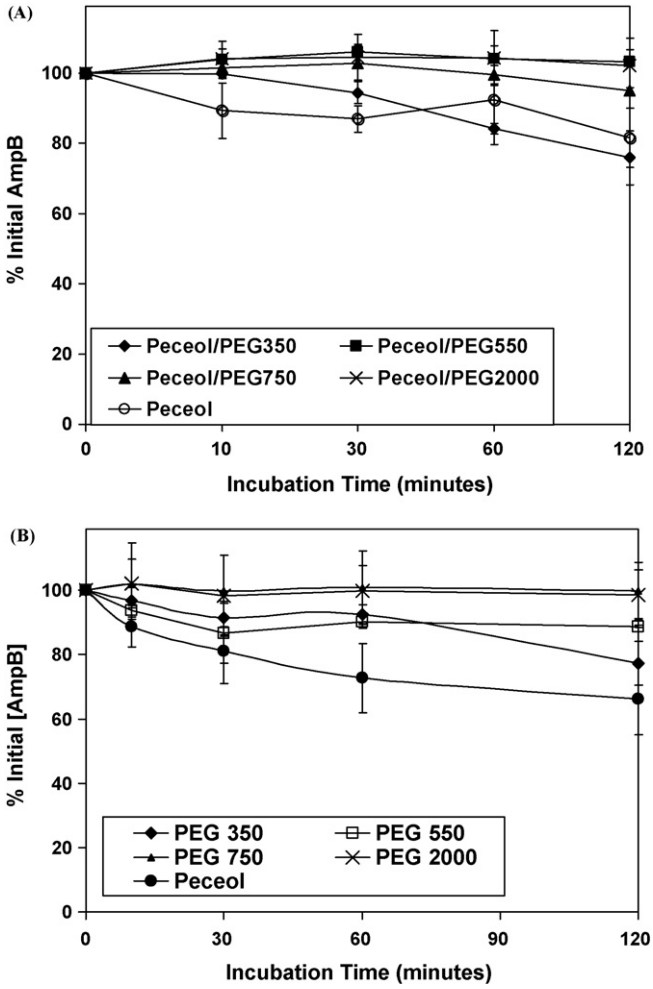

Fig. 4. Effect of PEG molecular weight on the stability of AmpB in fasted-state simulated intestinal fluid: (A) without lecithin and (B) with lecithin at 37 °C. Data represent the mean ± S.D. of three independent experiments, each of which was performed in triplicate.

shows representative images of AmpB in Peceol only (A) and in Peceol/DSPE-PEG2000 (B). AmpB/Peceol shows regional variations in nanometer-scale topography (a combination of both sample height and fluid stiffness) within individual droplets, whereas the

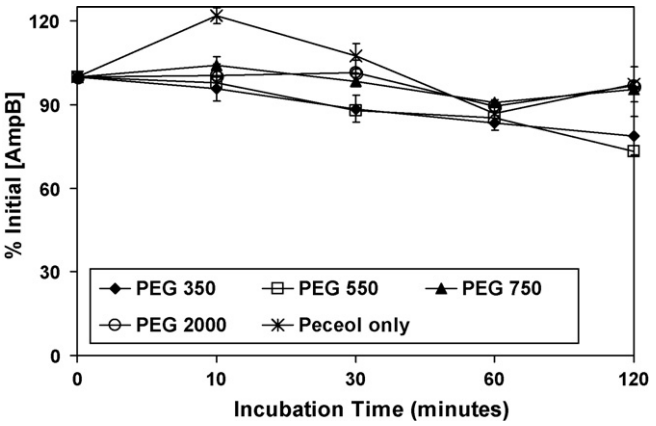

Fig. 5. Effect of PEG molecular weight on the stability of AmpB in fasted-state simulated intestinal fluid with pancreatin enzymes at 37 °C. Data represent the mean ± S.D. of three independent experiments, each of which was performed in triplicate.

AmpB/Peceol/DSPE-PEG2000 sample shows greater homogeneity and little or no crystalline features.

3.4. Antifungal activity and renal toxicity in rats infected with A. fumigatus and C. albicans

Peceol/DSPE-PEG2000-based oral AmpB treatment significantly decreased total fungal CFU concentrations recovered in all the organs added together by 80% compared to non-treated controls (Table 4) without significant changes in plasma creatinine levels (Table 5). Abelcet® treatment significantly decreased total fungal CFU concentrations recovered in all the organs added together by 88% compared to non-treated controls (Table 4) without significant changes in plasma creatinine levels (Table 5).

AmpB in Peceol/DSPE-PEG2000 administered orally significantly decreased kidney CFU concentrations by greater than 75% at the 5 mg/kg dose and 95% at the 10 mg/kg dose, respectively (Fig. 7) compared to non-treated controls without significant changes in plasma creatinine levels at the 5 mg/kg dose (data not shown) and at the 10 mg/kg dose (Table 6). No significant differences in other tissues were observed (data not shown). Abelcet® treatment significantly decreased kidney CFU concentrations by 98% compared to non-treated controls (Fig. 7) without significant changes in plasma creatinine levels (Table 6).

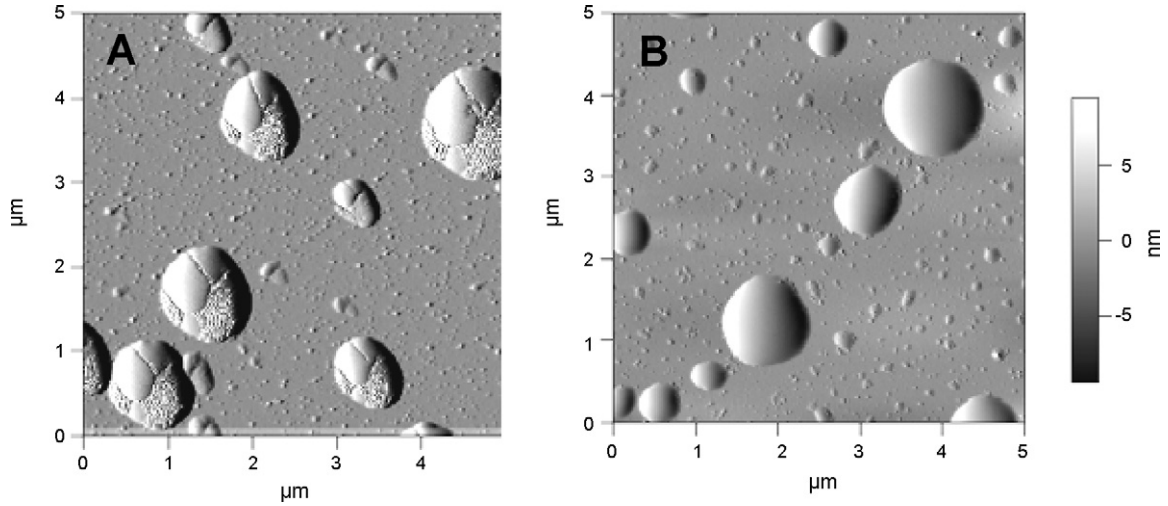

Fig. 6. Atomic force microscopy images of Amphotericin B prepared in (A) Peceol alone; (B) Peceol/DSPE-PEG2000. Droplets applied to the substrate exhibit phase separation of components in (A) but not in (B), demonstrating superior homogeneity of the Peceol/DSPE-PEG2000 formulation.

Table 4
Fungal analysis of *Aspergillus fumigatus*-infected male Sprague–Dawley rats treated with oral gavage doses of normal saline (non-treated control), Amphotericin-DSPE-PEG2000 incorporated into Peceol (10 mg/kg twice daily × 2 days) or a single intravenous dose of Abelcet® (ABLC; 5 mg/kg once daily × 2 days). All rats were infected with 2.9–3.45 × 10^7 viable colony forming units (CFU)/0.3 mL/rat of *Aspergillus fumigatus* prior to initiation of treatment.

Treatment groups	Infected tissues (CFU/mL of homogenized tissues)						
	Brain	Lungs	Heart	Liver	Spleen	Kidney	All organs
Non-treated controls (n = 9)	3538 ± 1810	74 ± 30	101 ± 63	308 ± 114	1163 ± 772	364 ± 119	5549 ± 2498
ABLC 5 (n = 4)	550 ± 445[a]	10 ± 4[a]	15 ± 3[a]	18 ± 5[a]	88 ± 44[a]	10 ± 0[a]	690 ± 419[a]
AmpB-DSPE-PEG2000 (n = 7)	736 ± 186[a]	51 ± 18	20 ± 4	180 ± 48	107 ± 32[a]	44 ± 10[a]	1139 ± 221[a]

Note: Previous studies have shown that AmpB alone does not have measurable accumulation at the doses used in this study. ABLC, Amphotericin B lipid complex.
[a] $p < 0.05$ vs. non-treated controls using Student t-test; all data are presented as mean ± S.E.M.

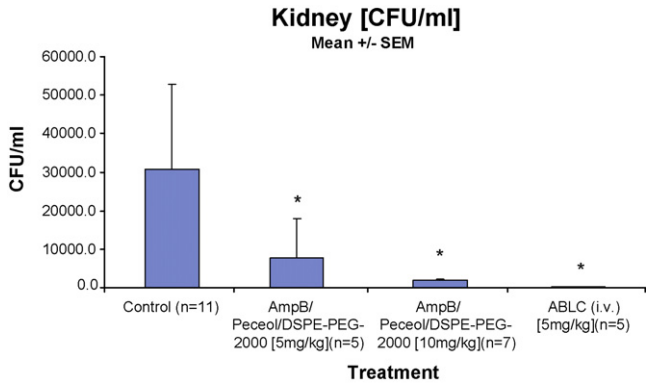

Fig. 7. Kidney fungal analysis of *Candida albicans*-infected male Sprague–Dawley rats treated with oral gavage doses of normal saline (non-treated control), Amphotericin-DSPE-PEG2000 incorporated into Peceol (5 and 10 mg/kg twice daily × 2 days) or a single intravenous dose of Abelcet® (ABLC; 5 mg/kg once daily × 2 days). All rats were infected with 2.9–3.45 × 10^7 viable colony forming units (CFU)/0.3 mL/rat of *Candida albicans* prior to initiation of treatment. Data is presented as mean ± S.E.M.; *$p < 0.05$ vs. untreated control.

Table 5
Plasma creatinine concentrations before infection (blank), pre-dose (0 h) and 48 h after treatment (48 h) in rats infected with *Aspergillius Fumigatus*.

Study groups	Plasma creatinine (mg/dL)		
	Blank	0 h	48 h
Saline-treated controls (n = 9)	0.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.2
AmpB in Peceol/DSPE-PEG2000 10mg/kg oral (n = 7)	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.1
ABLC 5mg/kg-IV (n = 4)	0.3 ± 0.2	0.4 ± 0.1	0.5 ± 0.1

Data presented as mean ±S.E.M.

Table 6
Plasma creatinine concentrations before infection (blank), pre-dose (0 h) and 48 h after treatment (48 h) in rats infected with *Candida albicans*.

Study groups	Creatinine (mg/dL)		
	Blank	0 h	48 h
Saline-treated controls (n = 9)	0.5 ± 0.3	0.5 ± 0.3	1.0 ± 0.5
AmpB in Peceol/DSPE-PEG2000 10mg/kg oral (n = 6)	0.6 ± 0.5	0.5 ± 0.5	0.5 ± 0.2
ABLC 5mg/kg IV (n = 4)	0.3 ± 0.3	0.4 ± 0.2	0.5 ± 0.2

Data presented as mean ± S.E.M.

4. Discussion

The administration of intravenous AmpB has been limited by its dose-dependent kidney toxicity that has not been predictable by monitoring plasma and/or serum drug concentration (Sachs-Barrable et al., 2008). A number of studies have reported that AmpB, solubilized in methanol, is poorly absorbed from the GI tract (Sachs-Barrable et al., 2008) and therefore is not commonly administered orally but intravenously, which can result in the aforementioned renal toxicity. However, to date, few studies investigating the development and assessing the antifungal activity of oral AmpB formulations have been reported.

The preliminary SEDDS formulations of Amphotericin B did produce self-emulsification and a small droplet size upon dispersion into simulated gastric fluid. However, due to the lack of physical stability, the maximum solubility that was achieved with this approach was only 0.5 mg/mL which is too low for in vivo use. AmpB in Peceol was stabilized and its solubility enhanced 50-fold by the incorporation of DSPE-PEG, where the PEG mean molecular weight was varied between 350 and 2000. The current study examined whether the difference in PEG MW would influence the stability of AmpB and/or its emulsification properties, as measured in simulated gastric and intestinal fluids similar to USP dissolution studies.

Drug stability in the stomach and intestine is critical for promoting drug absorption in the GI tract. AmpB is well known to be more soluble but relatively unstable at low pH, therefore any protection afforded by the lipid components of the formulation could be a significant benefit toward increasing the oral bioavailability of AmpB. It was also important to know if the lipidic vehicles influenced the superaggregation state of AmpB, which has been previously been shown to influence drug solubility as well as in vivo activity (Sánchez-Brunete et al., 2004; Tancrède et al., 1990). The UV spectral pattern of AmpB in the lipidic vehicles described here was consistent with monomeric AmpB before and after incubation in simulated gastric or intestinal fluids. No UV spectral pattern change was noted upon ambient temperature storage (21 °C) over a period of 4 weeks (data not shown). However, interactions between the AmpB and the lipid components in the undiluted formulation (in the absence of the assay solvent) or following oral absorption in vivo may be different.

Phase separation of components following temperature cycling could be a concern for multi-component lipidic formulations that can vary between semisolid and liquid within temperature ranges that correspond to seasonal ambient temperature variations. Phase separation could contribute to physical and chemical instability. Imaging the samples by atomic force microscopy demonstrated a clear difference between the behavior of lipid droplets composed of AmpB in Peceol vs. AmpB in Peceol/DSPE-PEG2000. The former sample had a much greater tendency to exhibit distinct subregions within the droplet consistent with crystals and phases each with a different force constant (or response) as observed in the AFM images (Fig. 6). These results suggest a stabilizing effect of DSPE-PEG2000 on the Peceol – a mixture of mono- and diglycerides – which was part of its intended purpose. AmpB in monoglyceride/DSPE-PEG2000 was not chemically stable at 43 °C for even several days (data not shown), further suggesting a potential role for the interaction between DSPE-PEG2000 and the diglyceride component of Peceol in the more stable AmpB/Peceol/DSPE-PEG2000, although this characteristic requires further study.

Stability in simulated gastric fluid over 2 h was excellent, with surprisingly little variability between formulations prepared with

the various DSPE-PEGs or with only Peceol. All showed a translucent appearance with no precipitate appearing. More variation in stability was observed in simulated fasted-state intestinal fluid containing bile salts. The emulsification properties of the bile salts, lecithin and phospholipase in pancreatin could influence formulation stability and therefore drug stability was evaluated in simulated intestinal fluids containing these components. Lecithin was likely incorporated into the lipid mixture when it was a component of the simulated intestinal fluid. As an emulsifier, lecithin would have the potential to either improve the association of Amphotericin B with the lipid excipients or to exclude it. The presence of lecithin, however, made no appreciable difference in the rate or extent of degradation or in the rank order of degradation at the end of 2 h. The influence of PEG MW on the AmpB formulation stability in the presence of lecithin-containing SIF was also of interest in terms of chemical degradation of AmpB. Clearly, DSPE-PEG 350 containing formulations provided less drug stability than those containing the longer-chain PEGs. In the absence of lecithin, submicron particle size analysis did not show a significant population below 50 nm that would be consistent with DSPE-PEG micelles, e.g. if DSPE-PEG350 had self-associated into a separate and excluded micelle population. Furthermore, there was no significant effect on emulsion droplet size (in fasted-state simulated intestinal fluid) due to the presence of DSPE-PEG or PEG MW, suggesting that the emulsification properties are largely derived from the Peceol component. It is possible that the improved stability of AmpB in Peceol/DSPE-PEG of higher MW may be related to the surface properties of the emulsion droplets themselves, in spite of the lack of a direct relationship to emulsion droplet size distribution itself. Theoretically, the hydrophilic PEG chains should orient to the water interface while the Peceol/AmpB fraction would remain in the inner oil phase and thereby sequestering and protecting the AmpB from degradation. This is consistent with the observations of increased stability for PEG MW = 750 and 2000 compared to 350 and 550. Furthermore, it is important to note that the interactions between AmpB and the Peceol/DSPE-PEG resulted in a UV spectral shape consistent with the more desirable monomeric AmpB rather than superaggregated AmpB (Fig. 2). The subject of whether AmpB in these formulations is actually in a lipid-associated solution vs. nanocrystalline suspension is presently under study.

We further hypothesized that the incorporation of AmpB into lipid-based formulations would have a major impact on the safety of this drug by altering AmpB's tissue distribution. Our previous findings suggested that AmpB incorporated into Peceol was less nephrotoxic than either intravenous or orally administered DOC-AmpB by decreasing the concentration of AmpB recovered in the kidney and increasing the concentration of AmpB recovered in the liver (Sachs-Barrable et al., 2008).

This report describes significant strides toward the development of a lipid-based Amphotericin B formulation for oral administration. A set of preliminary lipid-based AmpB oral formulations were prepared that provide solubilization and will serve as a formulation panel for testing in vitro drug uptake and in vivo bioavailability of oral AmpB. In particular, AmpB in Peceol/DSPE-PEG 2000 and Peceol/DSPE-PEG 750 appear to be the best candidates for further study due to their superior stability and solubilization of AmpB. The current study includes efficacy data such that further development and optimization of this oral AmpB formulation is now warranted by providing preliminary evidence that this new oral AmpB-Peceol formulation has antifungal activity without renal toxicity. Novel lipid-based AmpB oral formulations were prepared that provide excellent drug solubilization, drug stability in simulated gastric and intestinal fluids and antifungal activity without renal toxicity in rats infected with *A. fumigatus* and *C. albicans*.

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