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Assessing the Antifungal Activity of a New Oral Lipid-Based Amphotericin B Formulation Following Administration to Rats Infected with *Aspergillus Fumigatus*

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ABSTRACT The purpose of this study was to assess the antifungal activity of a new oral amphotericin B (AmpB) lipid-based formulation following administration to rats infected with *Aspergillus fumigatus*. *Aspergillus fumigatus* inoculum (2.1–2.5 × 10^7 colony forming units [CFU]) were injected via the jugular vein; 48h later male albino Sprague-Dawley rats (350–400 g) were administered either a single oral dose of AmpB incorporated into Peceol (50 mg AmpB/kg), physiologic saline (nontreated controls) or Peceol alone (vehicle control) once daily for 4 days. To assess antifungal activity Brain, Lung, Heart, Liver, Spleen and Kidney sections were homogenized with normal saline (1 mL/g of tissue) and a 0.1-mL aliquot was spread plated onto a Sabourand dextrose agar plate. The plates were incubated for 48 hr at 37°C, at which time the number of fungal CFU were determined and corrected for tissue weight. In addition, plasma galactomannan antigen concentrations were determined. Data was reported as mean ± standard error of the mean. The AmpB-Peceol oral formulation significantly decreased total fungal CFU concentrations recovered in all the organs added together, brain CFU concentrations, spleen CFU concentrations and plasma galactomannan antigen concentrations compared to baseline. No significant differences in lung, heart, liver and kidney CFU concentrations between treatment and control groups were observed. Peceol vehicle control did not exhibit any antifungal activity. These findings suggest that a new oral lipid-based formulation of AmpB incorporated into Peceol can significantly decrease brain and spleen CFU concentrations and plasma galactomannan antigen concentrations compared to non-treated controls.

KEYWORDS Amphotericin B, *Aspergillus fumigatus*, Oral formulation, Rats, Antifungal activity, Lipids

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INTRODUCTION

Despite the development of a number of new antifungal agents (Gates & Pinney, 1993), amphotericin B (AmpB) formulated as a micellar dispersion

(Fungizone®; Deoxycholate-AmpB [DOC-AmpB]), remains one of the most effective agents in the treatment of systemic fungal infections (Meyer, 1992). However, its use is often limited by dose-dependent renal toxicity (Gates & Pinney, 1993; Marr et al., 2004; Wasan et al., 1990; Wasan et al., 1994; Wasan et al., 1998). A number of studies have reported that monomeric AmpB, solubilized in methanol, is poorly absorbed from the gastrointestinal (GI) tract (Dangi et al., 1998; Lance et al., 1995; Souza et al., 2000) and therefore is not commonly administered orally but intravenously, which can result in the aforementioned renal toxicity.

Improved GI absorption of poorly absorbable drugs can be achieved by increasing the dissolution rate of the drug in the presence of bile acids. Within the GI tract, bile salts behave as biological detergents that when mixed with phospholipids form thermodynamically stable mixed micelles. Numerous studies have observed enhanced absorption of poorly absorbable drugs when administered as mixed micellar solutions (Hauss et al., 1998; Porter & Charman, 2001; Wasan, 2002). In addition, when AmpB was incorporated into mixed micelles containing bile acids and phospholipids it resulted in increased AmpB intestinal permeability and subsequent GI absorption using a rat intestinal perfusion methodology (Dangi et al., 1998). However, the limitation of this study was that various AmpB mixed micelle formulations were perfused through a cannulated upper intestine of an anesthetized rat. This model does not account for the effect of anesthesia and was not done in a whole animal model. Furthermore, the toxicological consequences of improving GI absorption were not investigated in this study, specifically, AmpB's dose-dependent renal toxicity, which limits the use of this compound.

Recently, our laboratory has determined that plasma AmpB levels were significantly elevated in rats following the oral administration of AmpB incorporated into Peceol (a lipid-based excipient composed of comprised primarily of a mixture of mono- and diglycerides of oleic acid, which closely resembles the end-products of intestinal lipid digestion (Hauss et al., 1998) compared to DOC-AmpB or AmpB solubilized in methanol (Risovic et al., 2003). We further observed that AmpB incorporated into Peceol was less renal toxic 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 and spleen (Risovic et al., 2003). However, to date, AmpB's antifungal activity following the administration of this new oral formulation has yet to be assessed.

Thus, the purpose of this study was to determine AmpB's antifungal activity following the administration of this new oral lipid-based AmpB formulation (100 mg of DOC-AmpB dissolved into 12 mL of 100% Peceol) to rats infected with *Aspergillus fumigatus*. Based on previous studies (Risovic et al., 2003) our working hypothesis was that incorporation of AmpB into Peceol would significantly enhance GI tract absorption resulting in increased plasma concentration and decreased organ colony forming units of *Aspergillus fumigatus* compared to non-treated controls.

METHODS AND MATERIALS

Male albino Sprague-Dawley rats (350–400 g) were purchased from Charles River. The rats were surgically implanted with a port (Access Technologies) and catheter with access to venous blood by a similar method used for rabbits (Wasan et al., 1998). Animals were housed in an animal facility with a 12 hr 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. The rat is an appropriate animal model to investigate the GI absorption of AmpB following oral administration due to similarities in intestinal characteristics (i.e., anatomical, metabolic and biochemical characteristics; Fagerholm et al., 1996; Kararli, 1995; Levet-Trafit et al., 1996; Soria & Zimmerman, 1996) between rats and humans.

Aspergillus fumigatus isolated from a patient with disseminated aspergillosis (provided by the BC Centre for Disease Control, F1048) was used to infect the rats. The inoculum was grown for 48 hr at 37°C on Sabouraud dextrose agar. Spores were harvested from the agar using glass beads and suspended in pyrogen-free saline. Spore suspensions were standardized to 4–5% transmission at 540 nm (LKB Ultraspec II). *Aspergillus fumigatus* inoculum ($2.1–2.5 \times 10^7$ colony forming units [CFU] determined by direct counting). Inoculum were injected *via* the jugular vein; 48 hr later male albino Sprague-Dawley rats (350–400 g) were administered

either a single oral dose of AmpB incorporated into Peceol (50 mg AmpB/kg; $n = 7$), physiologic saline (non-treated controls; $n = 7$) or a Peceol vehicle control ($n = 3$) once daily for 4 days.

The antifungal activity of AmpB was assessed by two different methods, organ CFU of *Aspergillus fumigatus* and by plasma galactomannan antigen detection. The choice of organ CFU as an indicator of antifungal activity was based on previously published work (Sivak et al., 2004). Brain, lung, heart, liver, spleen and kidney sections (1 g of each tissue) were homogenized with normal saline (1 mL; concentration of 1 g tissue/1 mL) (Heidolph diax 900). Ten-fold serial dilutions of 0.1 mL homogenate were spread plated onto duplicate Saboraud dextrose agar plates and incubated for 48 hr at 37°C. Surviving colonies of *A. fumigatus* were counted (mean CFU/mL homogenate, corrected for tissue weight).

Plasma galactomannan antigen detection was based on the work of Marr et al. (2004), which showed correlation between tissue fungal burden and galactomannan antigen index value. Using a commercially available sandwich ELISA kit (Platelia Aspergillus, Bio-Rad), rat plasma samples were processed as per manufacture's instructions. Briefly, 300µl of negative control, positive control, calibrator and unknown sample plasma (Blank, 0-, 48-, and 96-hr samples) were treated, incubated at 100°C for 3 min and centrifuged (10 min, 10000 *g*, room temperature). 50 µL of conjugate was added to the wells containing the antibodies to galactomannan. A total of 50 µL of the sample supernatant was added to the wells and incubated for 90 min at 37°C. The wells were then washed (5 times with diluted washing agent). A total of 200µL of the substrate buffer was added to each well and incubated in the dark for 30 min. The reaction was stopped with 100 µL 1.5 M sulfuric acid. Optical density was measured at 450 nm. Unknowns were compared to the calibrator (1 ng/mL) and an index value was calculated $\left(\frac{OD_{unknown}}{OD_{calibrator}}\right)$ and reported. An index value greater than 1 was considered to be positive for galactomannan antigen.

The numbers of CFUs in tissues and plasma galactomannan concentration were compared between each treatment group by student *t*-Test and ANOVA, respectively (INSTAT2; GraphPad Inc.). Critical differences were assessed by Tukey post hoc tests (Gates & Pinney, 1993). A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). Data was reported as mean ± standard error of the mean.

RESULTS AND DISCUSSION

Mean weight of rats was not significantly different prior to and following drug administration (data not shown). Similarly, kidney, liver, lung, spleen and heart weights were not different between control and drug treatment groups (data not shown). The AmpB-Peceol oral formulation significantly decreased total fungal CFU concentrations recovered in all the organs added together by 95% (351 ± 122 vs. 6759 ± 2706 CFU/mL of homogenized tissue; $p < 0.05$), brain CFU concentrations by 95% (221 ± 100 vs. 4499 ± 1757 CFU/mL of homogenized tissue; $p < 0.05$) and spleen CFU concentrations by 98% (27 ± 12 vs. 1763 ± 1544 CFU/mL of homogenized tissue; $p < 0.05$) compared to non-treated controls (Table 1). No significant differences in lung, heart, liver and kidney CFU concentrations between treatment and control groups were observed (Table 1). The AmpB-Peceol formulation

TABLE 1 **Fungal analysis of *Aspergillus fumigatus*-infected male Sprague-Dawley rats treated with single oral gavage doses of Normal Saline (nontreated control) or Amphotericin B incorporated into Peceol (100%) (50 mg/kg × 4 days). All rats were infected with 2.1–2.5 × 10^7 Viable Colony Forming Units (CFU)/0.3 mL/rat of *Aspergillus fumigatus* prior to initiation of treatment.**

	Infected Tissues (CFU/mL of homogenized tissues)						
Treatment Groups	Brain	Lungs	Heart	Liver	Spleen	Kidney	All Organs
Nontreated Controls (n = 7)	4499 ± 1757	139 ± 98	9 ± 5	269 ± 215	1763 ± 1544	81 ± 77	6759 ± 2706
Peceol-AmpB (n = 7)	221 ± 100[a]	50 ± 40	14 ± 6	27 ± 8	27 ± 12[a]	11 ± 10	351 ± 122[a]

[a] $p < 0.05$ vs. nontreated controls using student *t*-test; All data are presented as Mean ± SEM.

*Note: Previous studies have shown that AmpB alone does not have measurable accumulation at the doses used in this study.

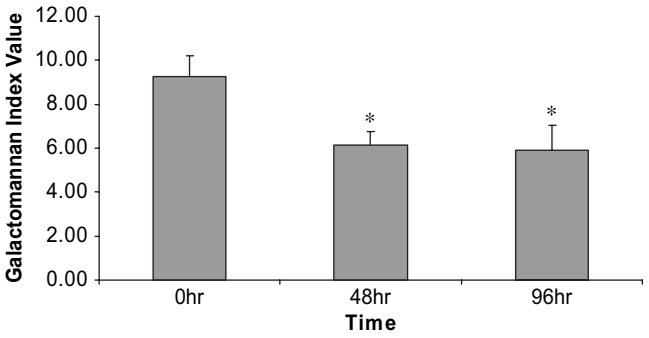

FIGURE 1 **Plasma galactomannan antigen concentrations (ng/mL) in *Aspergillus fumigatus*-infected male Sprague-Dawley rats prior to infection (pre-infection), predose (48 hr post-infection time 0), 48 and 96 hr following administration of a single oral gavage doses of Amphotericin B incorporated into Peceol (100%) (50 mg/kg × 4 days) $n = 6$. All rats were infected with $2.1–2.5 \times 10^7$ viable colony forming units (CFU)/0.3 mL/rat of *Aspergillus fumigatus* prior to initiation of treatment. *$p < 0.05$ vs. time 0 using ANOVA. *Note plasma galactomannan pre-infection is below the detectable limit of the assay.**

significantly decreased plasma galactomannan antigen levels by 38% (Fig. 1) while the un-treated control had only a 15% decrease in antigen levels over the same time period measured (data not shown). Peceol vehicle control did not exhibit any antifungal activity (data not shown).

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 (Gates & Pinney, 1993; Meyer, 1992; Wasan et al., 1998). A number of studies have reported that AmpB, solubilized in methanol, is poorly absorbed from the gastrointestinal (GI) tract (Dangi et al., 1998; Lance et al., 1995; Souza & Campa, 1999; Souza et al., 1993; Souza et al., 2000) 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.

A previous study published by our group reported considerable differences in the plasma concentration and tissue distribution of AmpB following administration of AmpB-Peceol compared to DOC-AmpB (Risovic et al., 2003). We hypothesized that this result may be explained by the fact that lymphatic transport of many water-insoluble drugs occurs concurrently with triglyceride absorption from the gastrointestinal tract (Hauss et al., 1998; Holm et al., 2002; Porter & Charman, 2001) and that Peceol could provide an efficiently absorbed source of lipid for promoting lymphatic drug transport, thus increasing systemic oral absorption of AmpB. Previous studies have demonstrated a significant increase in absorption of the hydrophobic drug, cyclosporine A, from "predigested" olive oil, when compared to a non-digested control (Reymond et al., 1988). Our group has recently published evidence suggesting that Peceol increases the gastrointestinal absorption of AmpB by increasing the amount of drug that is transported through the mesenteric lymph duct and by decreasing mdr-1 mRNA and p-glycoprotein (PGP) protein expression, resulting in lower PGP-mediated AmpB efflux (Risovic et al., 2004). However, additional studies to confirm these potential mechanisms are required.

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 renal toxic 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 (Risovic et al., 2003).

However, the main limitation of the previous AmpB-Peceol studies published by our group was the lack of any efficacy data to warrant further development and optimization of this oral AmpB formulation. The current study addresses this limitation by providing preliminary evidence that this new oral AmpB-Peceol formulation has antifungal activity. This study provides further justification of continuing to develop and optimize this novel oral lipid-based AmpB formulation.

In conclusion, our findings suggest that a new oral lipid-based formulation of AmpB incorporated into Peceol can significantly decrease brain and spleen CFU concentrations and plasma galactomannan antigen concentrations compared to non-treated controls.

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