Effect of Heat-Treated Amphotericin B on Renal and Fungal Cytotoxicity

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The purpose of this investigation was to determine the cytotoxicity of amphotericin B (AMB; trade name Fungizone [FZ]) following the administration of FZ and a heat-treated form of FZ (HFZ) to LLC-PK1 pig kidney cells and Cryptococcus neoformans var. gattii cells. HFZ was significantly less toxic to kidney cells than FZ at all concentrations tested. For both FZ and HFZ, the concentration range which resulted in a 50% reduction of the growth of fungal cells was 0.125 to 1 mg/ml. These findings suggest that heat treatment decreases AMB's renal cytotoxicity without modifying its antifungal activity.

Amphotericin B (AMB) is a polyene macrolide antibiotic used for the treatment of systemic fungal infections commonly found in immunocompromised patients (e.g., patients with AIDS), cancer patients, and diabetics (2, 4, 5, 12, 17, 23). The conventional AMB-deoxycholate micellar formulation Fungizone (FZ; Bristol-Myers Squibb, Princeton, N.J.) has been used for over 45 years, and despite its dose-dependent kidney toxicity, it remains the most widely used drug for the treatment of most systemic fungal infections (2, 12, 17). In addition, less-toxic liposomal and lipid-associated AMB formulations have been developed (e.g., AmBisome, Abeletes, also known as AMB lipid complex [ABLC] and Amphocil), and although they have proved to reduce AMB-induced kidney toxicity (7, 20–25), their use has been limited by their high expense.

A potentially simple and inexpensive alternative to the use of these formulations is the heat treatment (70°C for 20 min) of FZ to produce a “superaggregated” form of AMB, commonly referred to as heat-treated FZ (HFZ) (1, 6, 9, 14, 15). As reported by Hartsel et al., this new self-associated form of AMB is spectroscopically different from FZ, with a blue-shifted absorption maximum and a uniquely characteristic circular-dichroism spectrum (1, 9). Those authors further reported that this heat-induced superaggregated form of AMB was more stable in the presence of high- and low-density lipoproteins but that FZ was less stable and more dynamic, with the aggregate dissociating to a greater extent in the presence of either lipoprotein (9). Further studies using human monocytes indicated that HFZ provokes a smaller release of tumor necrosis factor alpha than FZ while retaining its AMB-induced antifungal activity (9, 16). It has been speculated that the nonaggregated form of AMB and the release of tumor necrosis factor alpha may be involved with side effects associated with AMB administration (1, 6, 9, 16).

Gaboriau et al. have reported that in vitro, HFZ is significantly less cytotoxic than FZ to human colon cancer cells but that its cytotoxic effect on Leishmania donovani is not diminished (6). In addition, Petit et al. have reported that HFZ has a therapeutic index superior to that of FZ in murine models of systemic candidiasis and leishmaniasis (14, 15). Recently, our group has reported that HFZ administration to rabbits significantly lowered AMB-induced renal toxicity and modified the pharmacokinetics of AMB in plasma and its distribution in tissue compared to those observed after FZ administration (11). However, to date, the results of few studies investigating AMB-induced renal cytotoxicity (the primary toxicity which limits AMB use) following HFZ administration versus that following FZ administration in a relevant cell culture model have been reported. In addition, few studies evaluating the effectiveness of HFZ versus that of FZ against Cryptococcus primary fungal isolates seen clinically have been carried out. Thus, the objective of this study was to evaluate the cytotoxicity of AMB following the administration of FZ, HFZ, and ABLC to LLC-PK1 pig kidney cells and cryptococcal strains from various sources. Our working hypothesis was that HFZ would be less cytotoxic to renal cells than FZ and would exhibit cytotoxicity equivalent to that of ABLC. In addition, we hypothesized that HFZ would be as toxic to fungal cells as FZ, AMB, and ABLC.

The commercially available lyophilized powder form of AMB-deoxycholate (FZ) was purchased from Bristol-Myers Squibb Canada Inc. For all toxicity and activity studies, a 100 μM solution of FZ in phosphate-buffered saline (pH 7.4) was prepared. HFZ was prepared by heating FZ solutions for 20 min in a water bath at 70°C as previously described (1, 6, 9). ABLC (Enzon Pharmaceuticals Inc.) was purchased from Vancouver General Hospital’s Department of Pharmacy Services. Pig kidney cells (LLC-PK1) were purchased from the American Type Culture Collection (Manassas, Va.). Dulbecco’s...
modified Eagle’s medium–Ham’s F-12 culture medium (1:1), Dulbecco’s phosphate buffered saline, penicillin-streptomycin (5,000 U of penicillin/ml, 5,000 µg of streptomycin/ml), and fetal calf serum (U.S. source) were purchased from Gibco BRL (Grand Island, N.Y.).

LLC-PK₁ cells were cultured in Dulbecco’s modified Eagle’s medium–Ham’s F-12 medium (1:1) containing 10% fetal calf serum, 100 U of penicillin/ml, and 100 µg of streptomycin/ml (22). Cells were grown to confluence at 37°C in humidified 5% CO₂ and subcultured every 48 h after a 1:2 split. For all experiments, LLC-PK₁ cells were seeded into 96-well plates at a density of 2 × 10⁴ cells per well in 200 µl of medium. Medium was exchanged for fresh medium every 24 h, and experiments were initiated approximately 48 h after seeding, when confluence was attained. The growth rates of the cells were not influenced by the addition of penicillin or streptomycin.

Cultures of Cryptococcus neoformans var. gattii were found to grow well in a semisynthetic medium, Difco yeast nitrogen base (formula (in amounts per liter): (NH₄)₂SO₄, 5 g; KH₂PO₄, 1 g; MgSO₄, 0.5 g; NaCl, 0.1 g; CaCl₂, 0.1 g; L-histidine monohydrochloride, 10 mg; L-d-methionine, 20 mg; L-d-tryptophan, 20 mg; inositol, 2 mg; boric acid, 0.5 mg; niacin, 0.4 mg; MnSO₄, 0.4 mg; pyridoxine HCl, 0.4 mg; ZnSO₄, 0.4 mg; thiamine HCl, 0.4 mg; Ca pantothenate, 0.4 mg; FeCl₃, 0.2 mg; Na₂MoO₄, 0.2 mg; riboflavin, 0.2 mg; p-aminobenzoic acid, 0.2 mg; KI, 0.1 mg; CuSO₄, 0.04 mg; folic acid, 2 µg; biotin, 2 µg]. The medium was reconstituted at 10× strength, supplemented with glucose (0.5 g/liter), filtered for sterilization (with a 0.22-µm pore-size filter; Corning catalog no. 430769), and stored at 4°C until it was used. For the assay, the medium was diluted with sterile distilled water; the final pH of the medium was 5.4 (8).

Medium was dispensed into sterile, lidded, flat-bottomed polystyrene microtiter plates (Corning catalog no. 3595). The first column of the plate was a negative control and contained medium only. The 2nd through 11th columns contained two-fold-decreasing dilutions of an antifungal agent, with the last column being in a sterile reagent reservoir (Corning Costar catalog no. 4870). All susceptibility tests were performed in triplicate in each experiment.

GATT strains were prepared by emulsifying isolated colonies in sterile 0.85% saline. The suspensions were standardized at an optical density at 550 nm of 1.0 absorbance unit (LKB Ultratrace II spectrophotometer) and were then diluted to yield an inoculum concentration of 10⁴ CFU/ml (final concentration). The concentrations of suspensions were checked by plate counting. The inocula were dispensed into the microtiter plates with an eight-channel micropipettor, with the culture suspension being in a sterile reagent reservoir (Corning Costar catalog no. 4870). All susceptibility tests were performed in triplicate in each experiment.

FZ, HFZ, and ABLC (5 to 100 µg of AMB per ml; n = 6 at each concentration) were incubated in 10% fetal calf serum-containing medium containing 2.0 × 10⁵ LLC-PK₁ cells per well. Cells were incubated with either no drug treatment (controls), FZ, HFZ, or ABLC (positive control) for 18 h at 37°C. To determine percent cytotoxicity, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) conversion assay was used to measure AMB toxicity in LLC-PK₁ cells as previously described (22).

Inoculated plates of C. neoformans var. gattii were incubated for 48 h at 35°C. Absorbance was determined at 420 nm with a SpectraMax microplate reader (Molecular Devices, Sunnyvale, Calif.). The plates were shaken for 5 s to suspend the cells prior to being scanned. Pilot studies performed with these cultures indicated that culture density was most reproducible at 48 h. The 50%-reduction-in-growth end point was defined as the lowest drug concentration resulting in a turbidity level that was ≈50% of the average turbidity untreated (control) wells (13). To assess the effective ranges of the activities of the antifungal agents, the lowest drug concentrations resulting in 20 and 80% reductions in growth turbidity compared to that of the growth control were also calculated. Percentages of cytotoxicity were compared between treatment groups by an unpaired t test (Instat; GraphPad). Critical differences were assessed by Tukey post hoc tests. A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% (P < 0.05). All data are expressed as means ± standard deviations.

FZ-induced toxicity to LLC-PK₁ cells increased in a concentration-dependent fashion (Fig. 1). No further increase in toxicity was observed at FZ concentrations greater than 100 µg/ml. The maximal toxicity observed with HFZ was significantly lower than that observed with FZ (Fig. 1). However, ABLC

**FIG. 1.** Dose-response effects of FZ and HFZ, shown as percentages of cytotoxicity of various concentrations of FZ and HFZ to LLC-PK₁ cells in tissue culture medium after 18 h of incubation at 37°C (n = 6). Values are means ± standard deviations. *, P < 0.05 compared with values for FZ.
had the lowest toxicity at all concentrations tested (data not shown). The vehicles used to solubilize FZ, HFZ, and ABLC exhibited minimal cytotoxicity (data not shown).

The relative toxicities of FZ, HFZ, and ABLC to *C. neoformans* var. *gattii* are shown in Fig. 2. The concentration range for both FZ and HFZ which resulted in a 50% reduction of growth was 0.125 to 1 μg/ml (mode = 1); for ABLC, the range was 0.03 to 0.25 μg/ml (mode = 0.125) (*P* < 0.01; Mann Whitney U test). All of the cultures tested shared the same range of values for 50% reduction in growth regardless of the source of the isolate (human, animal, or environmental). The concentration required for an 80% reduction in growth was equal to or within 1 dilution of the concentration required for a 50% reduction in growth for 100% of the cultures for all three antifungal agents. The concentration inhibiting 20% of the growth of the control was more varied for FZ and HFZ than for ABLC. The ranges of concentrations required for a 20% reduction in growth were 0.03 to 1 μg/ml (6 doubling dilutions) for FZ, 0.03 to 0.5 μg/ml for HFZ, and 0.03 to 0.125 μg/ml (3 doubling dilutions) for ABLC.

Our results demonstrate that HFZ is less toxic than FZ or AMB to LLC-PK1 pig kidney cells. The reduced level of HFZ toxicity may be explained by the presence of a superaggregated form of AMB following heat treatment. Previous studies have suggested that AMB exists in monomeric, oligomeric, and aggregated forms in aqueous solutions. The proportion of each form depends on the dilution and on the concentration and solvent of the stock solution (4, 5). The equilibrium between monomers and aggregates seems to play a key role in drug activity. The monomeric form binds to ergosterol in the fungal cell membranes and forms pores, whereas the self-associated form leads to the formation of transmembrane channels through the cholesterol-containing membranes. These pores induce an osmotic shock by a reversible leakage of electrolytes, associated at higher AMB concentrations with peroxidation of the membrane lipids, resulting in lysis of the cell. However, heat treatment of FZ creates a superaggregated form of AMB, which results in less cytotoxicity. It has been hypothesized that the superaggregated form of AMB does not form channels in the membrane, unlike the aggregated form, but exists near the membrane, releasing monomers of AMB which selectively permeate ergosterol-containing membranes (namely, fungal membranes) (4, 5).

Our results further demonstrate that HFZ is more toxic than or as toxic as FZ to a variety of cryptococcus fungal strains. It has been suggested that extracellular phospholipases produced by certain fungal strains may cause the disruption of HFZ into the active monomeric form of AMB, and as a consequence, these strains are just as susceptible to HFZ as they are to FZ (11). Swenson et al. (19) have previously demonstrated that extracellular lipases produced by certain strains of *Candida albicans* are able to hydrolyze the major lipid in ABLC, releasing active AMB, but that mutants of *C. albicans* that were resistant to ABLC in vitro were deficient in extracellular phospholipase production. The addition of exogenous phospholipase to the incubation medium of these strains restored their sensitivity to ABLC. However, studies to confirm that a similar phenomenon happens with HFZ are warranted. Our results are also consistent with studies that have shown a concentration dependence on the fungistatic and fungicidal action of AMB against cryptococcus (3, 10).

Results presented in this paper are consistent with results of in vivo studies. In those studies, we investigated the influence of prior heat treatment of FZ on AMB disposition, tissue distribution, and renal toxicity in rabbits and observed significantly lower increases in serum creatinine concentrations from baseline following HFZ administration than following FZ administration. This lack of change in serum creatinine concentrations indirectly suggested that HFZ does not damage the glomerular filtration of the kidney to the same extent that FZ does. In addition, we observed lower kidney AMB concentrations with a statistically significantly greater concentration of AMB in the liver following HFZ administration than following FZ administration. Gaboriau et al. have reported that HFZ...
exhibits significantly lower cytotoxicity than does FZ against other mammalian cells without its cytotoxic effect against fungal cells being diminished (6). Taken together, these findings suggest that the heat treatment of FZ into a superaggregated complex reduces the interaction with kidney cell membranes, resulting in lower cytotoxicity (1). In conclusion, we have demonstrated that HFZ is as cytotoxic to fungal cells as FZ but is less cytotoxic to renal cells.

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