Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 298 (2005) 211–218
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Note

Effect of heat-treatment and the role of phospholipases on Fungizone®-induced cytotoxicity within human kidney proximal tubular (HK-2) cells and *Aspergillus fumigatus*

Carlos Leon [a], Ross Taylor [a], Karen H. Bartlett [b], Kishor M. Wasan [a,*]

[a] *Division of Pharmaceutics and Biopharmaceutics, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, BC, Canada V6T 1Z3*
[b] *School of Occupational and Environmental Hygiene, The University of British Columbia, Vancouver, BC, Canada V6T 1Z3*

Received 2 March 2005; received in revised form 27 April 2005; accepted 27 April 2005

Abstract

The objectives of this study were to determine the effects of heat-treatment on Fungizone® (FZ)-induced cytotoxicity in human kidney (HK-2) cells and fungal isolates of *Aspergillus fumigatus*, and to determine the possible role of phospholipases (PLA_2 and PLC) on heat-treated FZ (HFZ)-associated renal cell toxicity. HK-2 cells were grown at 37 °C in T75 flasks and seeded in 96-well plates at 20,000 cells/well. FZ and HFZ concentrations of 10, 25 and 50 μg/mL of AmpB were prepared. Snake venom PLA_2 and PLC (2.15 U/mL) were pre-incubated with HFZ for 1 h prior to addition to the cells. After 18 h of incubation, an MTS assay was performed to assess cell viability through mitochondrial respiration. A spore suspension of *A. fumigatus* was prepared and 96-well plates were seeded at 500,000 spores/well. HFZ and FZ were prepared as above and incubated with the fungi at 35 °C. After 72 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of drug that inhibited visible growth. Student–Newman–Keuls multiple comparisons tests were conducted to determine statistical significance. FZ-induced cytotoxicity was significantly greater than for HFZ in HK-2 cells at amphotericin B (AmpB) concentrations between 10 and 50 μg AmpB/mL ($n = 5$–9, $p < 0.05$). HFZ and FZ were found to have similar minimum inhibitory concentration (MIC) ranges for *A. fumigatus* (0.225–0.25 μg) AmpB/mL; ($n = 6$). The addition of PLA_2 and PLC to 50 μg heat-treated AmpB/mL significantly enhanced the cytotoxicity compared to controls ($n = 6$, $p < 0.05$). The presence of the phospholipases did not alter FZ-associated renal cell toxicity. Taken together, these findings suggest heat-treatment significantly decreased FZ-induced cytotoxicity in HK-2 cells without altering toxicity against a reference strain of *A. fumigatus*. In addition, PLA_2 and PLC enhanced the renal toxicity associated with HFZ, but not that of FZ.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Heat-treated amphotericin B; Renal toxicity; Human kidney cells; *Aspergillus fumigatus*; Phospholipases; Fungizone®

* Corresponding author. Tel.: +1 604 822 4889; fax: +1 604 822 3035.
E-mail address: kwasan@interchange.ubc.ca (K.M. Wasan).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.04.018

1. Introduction

Amphotericin B (AmpB) is considered the drug of choice for the treatment of systemic fungal infections (Bodey, 1986; Dannaoui et al., 2002; Gates and Pinney, 1993; Ghannoum et al., 1992; Klesper et al., 1998; Meyer, 1992; Rothon et al., 1994). Nephrotoxicity is a major complication associated with its use, and appears to be related to higher cumulative doses, diuretic use, abnormal serum creatinine at baseline and the use of concomitant nephrotoxic drugs (Chavanet et al., 1994; Costa and Nucci, 2001; Gaboriau et al., 1997; Wasan and Conklin, 1997). The two major hypotheses for the pathogenesis of AmpB-related toxicities are direct effects of the drug on epithelial cell membrane and vasoconstriction (Meyer, 1992; Wasan et al., 1994; Zager, 2000). During the last few years, some randomized trials have tested different strategies to reduce AmpB-induced renal toxicity. These strategies include sodium supplementation, low-dose dopamine, slower infusion rates, the administration of AmpB in lipid emulsions, and in lipid-based formulations (Baas et al., 1999; Bodey, 1986; Chabot et al., 1989; Costa and Nucci, 2001). The results of these trials showed that the lipid formulations of AmpB significantly reduce nephrotoxicity. Unfortunately, these agents are costly, restricting their use to patients with a high risk of developing significant renal toxicity.

A potentially simple and inexpensive alternative to these costly formulations is the heat-treatment (70 °C for 20 min) of FZ to produce a "super-aggregated" form of FZ, referred as heat-treated FZ (HFZ) (Bartlett et al., 2004; Gaboriau et al., 1997; Kwong et al., 2001; Petit et al., 1998, 1999; Sivak et al., 2004; van Etten et al., 2000; Hartsel et al., 2001; Rogers et al., 2003). Studies designed to compare FZ and HFZ have determined that they present distinct molecular dichroism spectra (Gaboriau et al., 1997; van Etten et al., 2000). At the functional level, HFZ provokes a reduction in the release of tumor necrosis factor alpha in human monocytes (Gaboriau et al., 1997), which may be related to its reduced cytotoxicity. Gaboriau et al. (1997) have reported that HFZ exhibits significantly lower in vitro cytotoxicity against mammalian cells without affecting its cytotoxic effect against fungal cells.

Previous studies on HFZ, including work from our own group, has used a pig kidney cell line (Baas et al., 1999) or murine and rabbit models (Kwong et al., 2001; Petit et al., 1998; Sivak et al., 2004; van Etten et al., 2000), thus, stressing the importance of developing a human proximal tubular cell model to test the validity of the results obtained in these cell line and animal models. Studies assessing mechanisms of proximal tubular cell (PTC) physiology and pathophysiology increasingly utilize cell culture systems to avoid the complexity of whole organ/whole animal experiments. Furthermore, to date, investigating the utility of HFZ in a human cell line has not been completed.

Therefore, the objective of this study was to utilize a human proximal tubule cell line model for the analysis of AmpB-related nephrotoxicity. We used the HK-2 cell line (Ryan et al., 1994) which appears to be well-differentiated on the basis of its histochemical, immune cytochemical and functional characteristics, and it can reproduce experimental results obtained with freshly isolated PTCs. Moreover, HK-2 cells have been used to compare different liposomal AmpB formulations but not HFZ (Zager, 2000), establishing it as a valid model to study AmpB-dependent cytotoxicity in human proximal tubule cells. This work is the first study to compare FZ and HFZ in human proximal tubular cells and could help to determine if the reduced nephrotoxicity found in the pig, mouse and rabbit models following HFZ administration, may also be observed within a human cell line model. To determine the effect of the heat-treatment on the antifungal activity of the drug, we used an *Aspergillus* model. *Aspergillus fumigatus* is the most common invasive pathogen mold worldwide and accounts for the vast majority of infections (90%). Invasive aspergillosis has developed into a wide-spread life threatening fungal infection commonly found in immunocompromised patients (Bodey, 1986).

A further objective was to determine if the nephrotoxicity of AmpB can be enhanced by phospholipases A_2 and C as suggested by previous observations (Swenson et al., 1998) and our own preliminary results in LLC PK_1 cells, a pig proximal tubule cell model (data now shown).

2. Materials

The commercially available lyophilized powder form of AmpB-deoxycholate (Fungizone®; FZ) was purchased from Bristol-Myers Squibb Canada, Inc. For all toxicity and activity studies, a 100 μg/mL AmpB

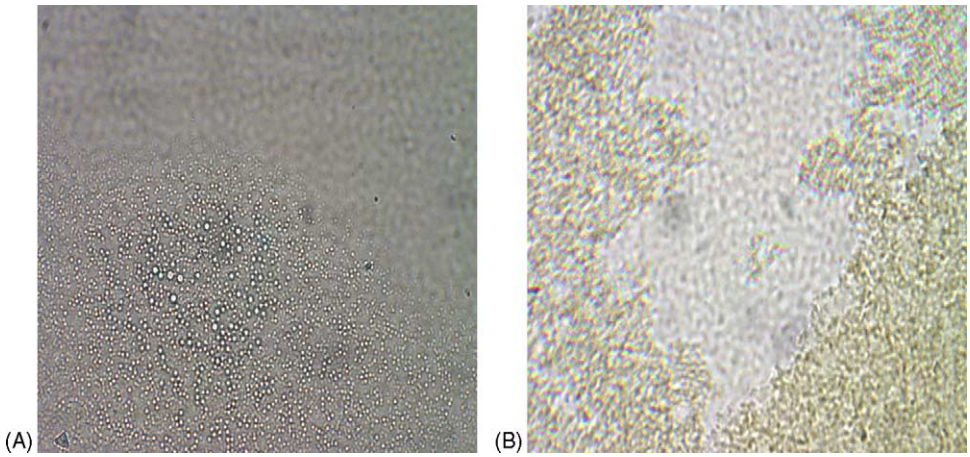

Fig. 1. Effects of heat-treatment on FZ. (A) FZ; (B) FZ after heated for 20 min at 70 °C. (×400).

solution in water was made. Heat-treated FZ (HFZ) was prepared by heating FZ solutions for 20 min in water-bath at 70 °C as previously described (Baas et al., 1999; Dannaoui et al., 2002). Aqueous solutions of FZ and HFZ (5 mg/mL in water) were visualized using light microscopy (Microscope: Olympus BX60, Media Cybernetics; software to capture the image: Spot 4.0.5 from Diagnostic Instruments Inc., MI) to confirm that heat-treatment resulted in aggregation of FZ (Fig. 1) as previously reported (Baas et al., 1999).

Human proximal tubule kidney cells (HK-2) were purchased from American type culture collection (Wanassas, VA). HK-2 cells were grown at 37 °C (5% CO_2) in T75 flasks with keratinocyte serum-free medium (K-SFM; Life Technologies, Grand Island, NY) containing 1 mM glutamine, 5 ng/mL epidermal growth factor, 40 μg/mL of bovine pituitary extract, 25 U/mL penicillin and 25 μg/mL of streptomycin. Dulbecco's phosphate buffered saline and penicillin–streptomycin (5000 U/mL penicillin, 5000 μg/mL streptomycin) were purchased from Life Technologies (Grand Island, NY).

3. Experimental methods

At near confluence, the cells were trypsinized and transferred to either additional T75 flasks (for passage) or to 96-well plates (seeded at a density of 20,000 cells/well) for the specific experiments. Experiments were conducted 48 h after passage, with the cells in a subconfluent state. FZ and HFZ (5–100 μg of AmpB/mL; quadruplicate measurements per concentration, $n = 5$–9) diluted in keratinocyte medium were added to the wells containing the HK-2 cells. Cells were incubated with either no drug treatment (controls), FZ (positive control) or HFZ for 18 h at 37 °C. Snake venom PLA_2 (2.15 U/mL) or *Clostridium perfringens* phospholipase C (2.15 U/mL) (both from Sigma–Aldrich, St. Louis, MI) were pre-incubated with HFZ in Keratinocyte complete medium for 1 h prior to addition to the cells. After 18 h of incubation, an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay was performed to assess cell viability through mitochondrial respiration as has been previously used in HK-2 cells (Ryan et al., 1994; Somji et al., 2004).

Percent AmpB-mediated cytotoxicity was determined using the MTS conversion assay (Promega Corporation, Madison, WI), as previously described (Zager, 2000).

An *A. fumigatus* reference strain (ATCC 9197; American Type Culture Collection (Wanassas, VA) was grown on BBL™ Sabouraud Dextrose Agar. Briefly, inoculated plates were incubated for 48 h at 35 °C. A spore suspension of *A. fumigatus* was prepared in sterile PBS with 0.1% Tween 20. The fungal concentration was calculated by three methods: counting under the microscope using a haematocytometer, measuring transmittance in a spectrophotometer and plating fungal dilutions in BBL™ Sabourand Dextrose

Agar plates to determine colony forming units. The fungi were seeded in 96-well plates at a concentration of 500,000 spores/well in RPMI-1640-MOPS medium (NCCLS, 2002). Medium was dispensed into sterile, lidded, flat-bottomed polystyrene microtitre plates (Fisher Scientific, XXX). The first columns contained decreasing dilutions of antifungal agent ranging from 10 to 0.1 μg/mL final concentration. The last column contained only inoculum and medium to serve as the growth control. The plates were placed in a 35 °C incubator for 72 h. At the end of this period, the MIC (minimal inhibitory concentration) was determined. The MIC was defined as the lowest drug concentration that prevents any discernible growth (NCCLS, 2002).

Percentage of cytotoxicity were compared between treatment groups by an unpaired *t*-test (INSTAT, GraphPad). Student–Newman–Keuls multiple comparisons tests were conducted to determine statistical significance, defined as 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.

4. Results and discussion

Fig. 1 is a picture of AmpB-micelle suspension (Fungizone®) after heat-treatment using light microscopy. The figure illustrates that the AmpB-micelle suspension (seen in Fig. 1A) forms aggregated clumps (Fig. 1B) following heat-treatment as reported previously (Gaboriau et al., 1997).

After determining a physical change in HFZ compared to FZ, we set as a goal to determine the effect of heat-treatment in cytotoxicity using an HK-2 cell model. AmpB-induced toxicity to HK-2 cells increased in a concentration-dependent fashion (Fig. 2). Both at a high AmpB concentration (100 μg AmpB/mL) and at a low AmpB range (5 μg AmpB/mL) there was no difference between FZ and HFZ. In the range of 10–50 μg AmpB/mL, the toxicity observed with HFZ was significantly lower than that observed with FZ (Fig. 2, $p < 0.05$). The vehicles used to solubilize FZ and HFZ exhibited minimal cytotoxicity (data not shown). Our results demonstrate that HFZ is less toxic that FZ to the HK-2 proximal tubule cells. FZ-induced cytotoxicity at 50 μg AmpB/mL was significantly greater than for HFZ in HK-2 cells (71.29 ± 10.23% versus 35.45 ± 8.79% cytotoxicity) (Fig. 2, $n = 8$, $p < 0.05$).

The reduced level of HFZ toxicity may be explained by the presence of a super "aggregated" form of AmpB following heat-treatment. Previous studies have suggested that AmpB exist 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

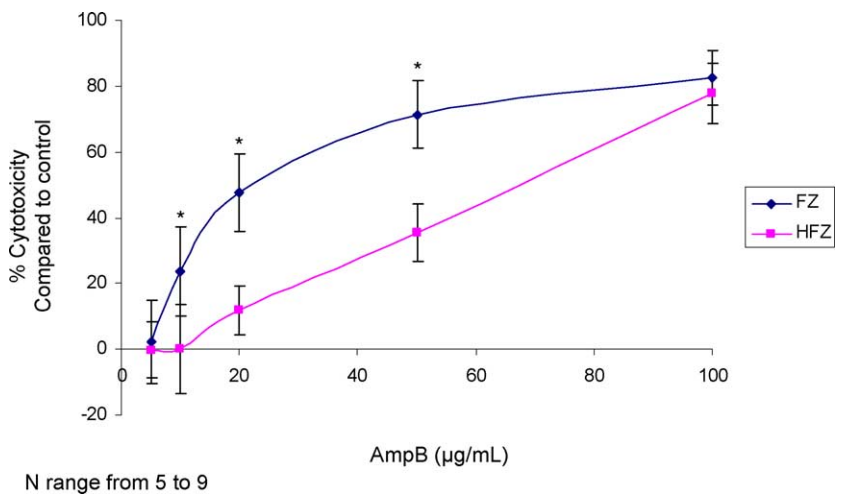

Fig. 2. Dose–response effects of FZ and HFZ. Percent cytotoxicity of HK-2 cells in tissue culture medium after 18 h of incubation at 37 °C with various concentrations of Fungizone (FZ) and heat-treated Fungizone (HFZ). Data are reported as means ± standard deviation of five to nine independent experiments (quadruplicate determinations per experiment) $^{*}p < 0.05$ vs. HFZ.

(Gaboriau et al., 1997). 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 AmpB concentrations with peroxidation of the membrane lipids, resulting in lysis of the cell. However, heat-treatment of FZ creates a super-aggregated form of AmpB, which results in less cytotoxicity. It has been hypothesized that the "super-aggregated" form of AmpB does not form channels in the membrane, unlike the aggregated form, but exists near the membrane, releasing monomers of AmpB which selectively porates ergosterol-containing membranes (namely fungal membranes) (Burgess and Hastings, 2000; Chabot et al., 1989; Reeves et al., 2004). The super-aggregated form of AmpB refers to form that does not release monomers of AmpB unlike the aggregated form.

However, this explanation is insufficient on its own to account for the similar level of efficacy of FZ and HFZ that we have found in an in vitro *Aspergillus* model. The relative toxicity of FZ and HFZ for *A. fumigatus* was determined in vitro. The concentration range which resulted in a 100% reduction of growth for both FZ and HFZ was 0.225–0.25 μg AmpB/mL (mode = 1) ($n = 6$, $p < 0.05$ Mann–Whitney U). The effective concentration range of FZ for *A. fumigatus* is considerably lower than in HK-2 cells for the following reason. AmpB acts by forming pores with the ergosterol contained in the membranes of fungi, but it also interacts with the cholesterol contained in the membranes of eukaryotic cells, hence its toxicity. However, AmpB has a far greater affinity for ergosterol (fungi containing sterol) than cholesterol (mammalian containing sterol) and, thus, requires far less drug to cause cytotoxicity. The observation that the efficacy of Fungizone was not affected by heating is consistent with previous reports in *Zygomycota* (Dannaoui et al., 2002) and *Candida* (van Etten et al., 2000), including our own work on *Cryptococcus* (Bartlett et al., 2004). Our results and the cited literature support the consideration of HFZ as a drug with a potentially higher therapeutic index than FZ.

To reconcile the similar levels of efficacy of FZ and HFZ with the physical and toxicological properties of FZ and HFZ, we suggest the possibility of fungal phospholipases which can "disaggregate" the complexed AmpB present in heat-treated FZ form, and thus, enhance its toxicity to similar levels of FZ. The production of extracellular phospholipases has been shown to be important in the pathogenesis of several bacterial infections, such as *Listeria* and *Pseudomonas* (Meyers et al., 1992). Increased phospholipase activity has also been correlated to increased mucosal pathogenicity in the yeast *Candida* (Barrett-Bee et al., 1985). The secretion of PLC has been shown to permeabilize the membrane of mammalian cells to allow the entry of protein toxins (Otero and Carrasco, 1988). In relation to our *Aspergillus* model, a fast atom bombardment technique was used (Birch et al., 1996) to determine the presence of *A. fumigatus* phospholipases in culture medium. The presence of anions corresponding to the phospholipids breakdown products indicated the presence of phospholipases A_1 and A_2, B, C and D. There is a report that suggests that AmpB mediates membrane permeabilization and release of proteins from *Aspergillus* (Reeves et al., 2004). This observation is consistent with the possibility that in the presence of the drug and the fungi, fungal proteins such as phospholipases are released and the cytotoxicity is enhanced. In our study, we show that HFZ is as toxic as FZ to an *A. fumigatus* reference strain. It has been suggested that extracellular phospholipases produced by certain fungal strains may cause the disruption of HFZ into the active monomeric form of AmpB and as a consequence these strains are just as susceptible to HFZ as they are to FZ (Swenson et al., 1998). Swenson et al. have previously demonstrated that extracellular lipases produced by certain strains of *Candida albicans* are able to hydrolyze the major lipid in ABLC, releasing active AmpB, while 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 (Swenson et al., 1998). Their data also supports the possibility that a host phospholipase may be secreted to the medium and enhance the cytotoxic effects of AmpB. PLA_2 can be found in circulating plasma (Kortesuo et al., 1992) ranging from 2 U/L in healthy individuals to 500 U/L in patients with severe infections and neoplasms (Aufenanger et al., 1993; Kortesuo et al., 1992). Studies to confirm that circulating PLA_2 alter HFZ nephrotoxicity in vivo are

warranted. Our results are also consistent with studies that have shown a concentration dependence on the fungistatic and fungicidal action of amphotericin B against *Aspergillus* (Sivak et al., 2004).

At this point, it is important to mention that our observations point to a possible role of phospholipases A_2 and C on mediating the AmpB-related nephrotoxicity. Independently, PLA_2 and PLC had no apparent nephrotoxic effect on the HK-2 cells; however, the addition of 2.15 U/mL of PLA_2 to 50 μg heated-AmpB/mL significantly enhanced the cytotoxicity ($46.34 \pm 10.72\%$ versus $21.46 \pm 6.918\%$ cytotoxicity) (Fig. 3, $n = 6$, $p < 0.05$). The effect of PLC on HFZ-mediated cytotoxicity was even more dramatic ($75.41 \pm 13.83\%$ versus $21.46 \pm 6.918\%$) increasing it to those levels found with a similar dose of FZ ($64.77 \pm 9.316\%$). Interestingly, both phospholipases did not enhance the cytotoxicity mediated by FZ at the doses tested (data not shown), suggesting that, at this dose, their effect is specific to HFZ. When the HK-2 cells were exposed to phospholipases A_2 and C, there was rather a slight increase in the respiration values. In the case of PLA_2, this is consistent with previous findings where this phospholipase protects mouse cells from nephrotoxicity (Zager et al., 1996). Our results cannot exclude the possibility that a host phospholipase interacts with the drug or the membrane may contribute to its enhancement of AmpB cytotoxicity.

At this moment, our data do not distinguish between two plausible possibilities: (a) that these phospholipases (host or fungal) have mainly a direct effect on HFZ or (b) a synergistic effect of the phospholipases and AmpB at the level of the membrane. Both possibilities merit further investigation and they could be addressed by the use of specific irreversible inhibitors of these phospholipases to be added post the incubation with the drug and prior to the addition of the AmpB–phospholipase complex to the cells. A study by Gottfredsson et al., tested the effect of deleting phospholipase B in *C. albicans* and *C. neoformans* and the susceptibility to AmpB (Gottfredson et al., 2001). They did not find a significant difference between the phospholipase B deficient fungi and the control fungi in their drug susceptibility, which suggests that phospholipase B does not contribute to

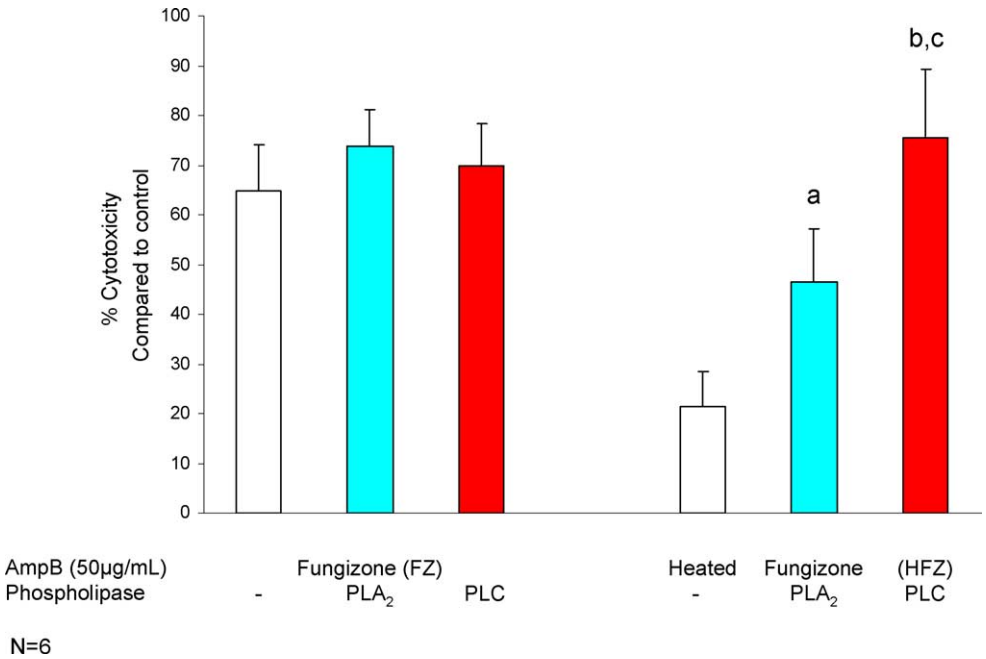

Fig. 3. Percent cytotoxicity of HK-2 cells in tissue culture medium after 18 h of incubation at 37 °C with Fungizone (FZ) and heat-treated Fungizone (HFZ) +/− phospholipase A2 or C. Data are reported as means ± standard deviation of six independent experiments (quadruplicate determinations per experiment). (a) $p < 0.05$ vs. heat-treated Fungizone (HFZ); (b) $p < 0.01$ vs. HFZ; (c) $p < 0.05$ vs. HFZ + PLA_2.

the AmpB susceptibility. These results are not in contradiction with our results, since we have not tested the phospholipase B effects. To our knowledge, there is no literature on *A. fumigatus* PLA_2 or PLC mutants and the role of these enzymes on AmpB susceptibility. There is only one study on the enhancement of platinum-drug cytotoxicity in a human carcinoma cell line by liposomal AmpB and phospholipase A_2 (Ferguson et al., 1999). In this study liposomal AmpB enhanced the cellular uptake of cisplatin; the liposomal AmpB plus phospholipase A_2 group showed the highest cisplatin cytoxicity. The authors suggest that this phospholipase may destabilize the liposome, allowing free AmpB to be released.

Results presented in this paper regarding the reduced nephrotoxicity of HFZ versus FZ are consistent with those we observed in vivo. In those studies (Kwong et al., 2001), we investigated the influence of prior heat-treatment of FZ on AmpB disposition, tissue distribution and renal toxicity in rabbits and observed significantly lower increases in serum creatinine concentrations from baseline following HFZ administration than FZ administration. This lack of change in serum creatinine concentrations indirectly suggested that at the concentration tested, HFZ does not damage the glomerular filtration of the kidney to the same extent FZ does. In addition, we observed lower AmpB kidney concentrations with a statistically significant greater concentration of AmpB in the liver following HFZ than FZ administration. We further have reported that HFZ retains AmpB antifungal activity with significantly less AmpB-induced renal toxicity compared to FZ following administration to rats infected with *A. fumigatus* (Sivak et al., 2004). Gaboriau et al. (1997) have reported that HFZ exhibits significantly lower cytotoxicity against other mammalian cells compared to FZ without diminishing its cytotoxic effect against fungal cells. Taken together, these findings suggest that the heat-treatment of FZ into a "super-aggregated" complex has reduced interaction with kidney cell membranes resulting in lower cytotoxicity (Baas et al., 1999). In conclusion, we have demonstrated that HFZ is equally cytotoxic to fungal cells but less cytotoxic to renal cells compared to FZ. We have also shown that phospholipases may enhance the nephrotoxic effect of HFZ in a human proximal tubule cell model that could be useful in developing novel AmpB formulations.

Acknowledgements

This project was funded by the Canadian Institute of Health Research Operating Grant (#MOP-14484 to K.M.W.). The study was supported in part by the Michael Smith Foundation for Health Research (K.H.B.). We thank Steve Lee for his assistance in the statistical analysis.

References

Aufenanger, J., Zimmer, W., Kattermann, R., 1993. Characteristics and clinical application of a radiometric *Escherichia coli*-based phospholipase A2 assay modified for serum analysis. Clin. Chem. 39, 605–613.

Baas, B., Kindt, K., Scott, A., Scott, J., Mikulecky, P., Hartsel, S.C., 1999. Activity and kinetics of dissociation and transfer of amphotericin B from a novel delivery form. AAPS PharmSci. 1, 1–11.

Barrett-Bee, K., Hayes, Y., Wilson, R.G., Ryley, J.F., 1985. A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J. Gen. Microbiol. 131, 1217–1221.

Bartlett, K.H., Yau, E., Hartsel, S.C., Hamer, A., Tsai, G., Bizzotto, D., Wasan, K.M., 2004. Effect of heat-treated amphotericin B on renal and fungal cytotoxicity. Antimicrob. Agents Chemother. 48, 333–336.

Birch, M., Robson, G., Law, D., Denning, D.W., 1996. Evidence of multiple extracellular phospholipase activities of *Aspergillus fumigatus*. Infect. Immun. 64, 751–755.

Bodey, G.P., 1986. Infection in cancer patients: a continuing association. Am. J. Med. 81 (11), 26.

Burgess, D.S., Hastings, R.W., 2000. A comparison of dynamic characteristics of fluconazole, itraconazone, and amphotericin B against *Cryptococcus nroformans* using time-kill methodology. Diagn. Microbiol. Infect. Dis. 38, 87–93.

Chabot, G.G., Pazdur, R., Valeriote, F.A., Baker, L.H.H., 1989. Pharmacokinetics and toxicity of continuous infusion of amphotericin B in cancer patients. J. Pharm. Sci. 78, 307–310.

Chavanet, P., Joly, V., Rigaud, D., Bolard, J., Carbon, C., Yenni, P., 1994. Influence of diet on experimental toxicity of amphotericin B deoxycholate. Antimicrob. Agents Chemother. 38, 963–968.

Costa, S., Nucci, M., 2001. Can we decrease amphotericin nephrotoxicity? Curr. Opin. Crit. Care 7, 379–383.

Dannaoui, E., Meis, J.F.G.M., Mouton, J.W., Verweij, P.E., 2002. In vitro susceptibilities of zygomycota to polyenes. J. Antimicrob. Chemother. 49, 741–744.

Ferguson, P.J., Currie, C., Vincent, M.D., 1999. Enhancement of platinum-drug cytoxicity in a human head and neck squamous cell carcinoma line and its platinum-resistant variant by liposomal amphotericin B and phospholipase A2-II. Drug Metab. Dispos. 27, 1399–1405.

Gaboriau, F., Cheron, M., Petit, C., Bolard, J., 1997. Heat-induced superaggregation of amphotericin B reduces its in vitro toxicity: a new way to improve its therapeutic index. Antimicrob. Agents Chemother. 41, 2345–2351.

Gates, C., Pinney, R.J., 1993. Amphotericin B and its delivery by liposomal and lipid formulations. J. Clin. Pharm. Ther. 18, 147–153.

Ghannoum, M.A., Ibrahim, A.S., Fu, Y., Shafiq, M.C., Edwards Jr., J.E., Criddle, R.S., 1992. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. J. Clin. Microbiol. 30, 2881–2886.

Gottfredson, M., Jessup, C.J., Cox, G.M., Perfect, J.R., Ghannoum, M.A., 2001. Fungal phospholipase activity and susceptibility to lipid preparations of amphotericin B. Antimicrob. Agents. Chemother. 45, 3231–3233.

Hartsel, S.C., Baas, B., Bauer, E., Foree, L.T., Kindt, K., Preis, H., Scott, A., Kwong, E.H., Ramaswamy, M., Wasan, K.M., 2001. Heat-induced superaggregation of amphotericin B modifies its interaction with serum proteins and lipoproteins and stimulation of TNF-a. J. Pharm. Sci. 90, 124–133.

Kwong, E.H., Ramaswamy, M., Bauer, E.A., Hartsel, S.C., Wasan, K.M., 2001. Heat treatment of amphotericin B modifies its serum pharmacokinetics, tissue distribution, and renal toxicity following administration of a single intravenous dose to rabbits. Antimicrob. Agents Chemother. 45, 2060–2063.

Klesper, M.E., Wolfe, E.J., Pfaller, M.A., 1998. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B against *Cryptococcus neoformans*. J. Antimicrob. Chemother. 41, 397–401.

Kortesuo, P.T., Nevalainen, T.J., Buchler, M., Uhl, W., 1992. Characterization of two phospholipases A2 in serum of patients with sepsis and acute pancreatitis. Eur. J. Clin. Chem. Clin. Biochem. 30, 263–269.

Meyer, R.D., 1992. Current role of therapy with amphotericin B. Clin. Infect. Dis. 14, S154–S160.

Meyers, D.J., Palmer, K.C., Bale, L.A., Kernacki, K., Preston, M., Brown, T., Berk, R.S., 1992. In vitro and in vivo toxicity of phospholipase C from *Pseudomonas aeruginosa*. Toxicon 30, 161–169.

NCCLS, 2002. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard, second ed. NCCLS document M27-A2 (ISBN 1-56238-469-4) NCCLS, Wayne, PA.

Otero, M.J., Carrasco, L., 1988. Exogenous phospholipase C permeabilizes mammalian cells to proteins. Exp. Cell Res. 177, 154–161.

Petit, C., Cheron, M., Joly, V., Rodrigues, J.M., Bolard, J., Gaboriau, F., 1998. In-vivo therapeutic efficacy in experimental murine mycoses of a new formulation of deoxycholate-amphotericin B obtained by mild heating. J. Antimicrob. Chemother. 42, 779–785.

Petit, C., Yardley, V., Gaboriau, F., Bolard, J., Croft, S.L., 1999. Activity of a heat-induced reformulation of amphotericin B deoxycholate (fungizone) against *Leishmania donovani*. Antimicrob. Agents Chemother. 43, 390–392.

Reeves, E.P., Murphy, T., Daly, P., Kavanagh, K., 2004. Amphotericin B enhances the synthesis and release of the immunosuppressive agent gliotoxin from the pulmonary pathogen *Aspergillus fumigatus*. J. Med. Microbiol. 53, 719–725.

Rogers, P.D., Barker, K.S., Herring, V., Jacob, M., 2003. Heat-induced superaggregation of amphotericin B attenuates its ability to induce cytokine and chemokine production in human monocytic cell line THP-1. J. Antimicrob. Agents Chemother. 51, 405–408.

Rothon, D.A., Mathias, R.G., Schechter, M.T., 1994. Prevalence of HIV infection in provincial prisons in British Columbia. Can. J. Med. Assoc. 151, S154–S160.

Ryan, M.J., Johnson, G., Kirk, J., Fuerstenberg, S.M., Zager, R.A., Torok-Storb, B., 1994. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. Kidney Int. 45, 48–57.

Sivak, O., Bartlett, K., Wasan, K.M., 2004. Heat-treated fungizone retains amphotericin B antifungal activity without renal toxicity in rats infected with *Aspergillus fumigatus*. Pharm. Res. 21, 1564–1566.

Somji, S., Garrett, S.H., Sens, M.A., Gurel, V., Sens, D.A., 2004. Expression of metallothionein isoform 3 (MT-3) determines the choice between apoptotic or necrotic cell death in Cd+2-exposed human proximal tubule cells. Toxicol. Sci. 80 (2), 358–366.

Swenson, C.E., Perkins, W.R., Roberts, P., Ahmad, I., Stevens, R., Stevens, D.A., Janoff, A.S., 1998. In vitro and in vivo antifungal activity of amphotericin B lipid complex: are phospholipases important? Antimicrob. Agents Chemother. 42, 767–771.

van Etten, E.W., van Vianen, W., Roovers, P., Frederik, P., 2000. Mild heating of amphotericin B-desoxycholate: effects on ultrastructure, in vitro activity and toxicity, and therapeutic efficacy in severe candidiasis in leukopenic mice. Antimicrob. Agents Chemother. 44, 1598–1603.

Wasan, K.M., Rosenblum, M.G., Cheung, L., Lopez-Berestein, G., 1994. Influence of lipoproteins on renal cytotoxicity and antifungal activity of amphotericin B. Antimicrob. Agents Chemother. 38, 223–227.

Wasan, K.M., Conklin, J.S., 1997. Enhanced Amphotericin B nephrotoxicity in intensive care patients with elevated levels of low-density lipoprotein cholesterol. Clin. Infect. Dis. 24, 78–80.

Zager, R.A., Burkhart, K.M., Conrad, D.S., Gmur, D.J., Iwata, M., 1996. Phospholipase A2-induced cytoprotection of proximal tubules: potential determinants and specificity for ATP depletion-mediated injury. J. Am. Soc. Nephrol. 7, 64–72.

Zager, R.A., 2000. Polyene antibiotics: relative degrees of in vitro cytotoxicity and potential effects on tubule phospholipid and ceramide content. Am. J. Kidney Dis. 36, 238–249.