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Mixed Micellar Nanoparticle of Amphotericin B and Poly Styrene-block-poly Ethylene Oxide Reduces Nephrotoxicity but Retains Antifungal Activity

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Mixed micellar nanoparticle consisting of amphotericin B (AmB) and poly styrene-block-poly ethylene oxide (PS-block-PEO) was prepared by high pressure homogenizer. Nephrotoxicity of the nanoparticle was investigated along with antifungal activity and self-aggregation status of the drug in the nanoparticle. Nephrotoxicity was markedly reduced when AmB was intravenously administered to rats as mixed micellar nanoparticle with PS-block-PEO in terms of transmission electron microscopy of tubular cells and creatinine clearance. Antifungal activity of AmB was not altered when the drug was in the form of mixed micellar nanoparticle compared to both conventional formulation and AmB micelle treated by same procedure without PS-block-PEO. Self-aggregation status of AmB molecules revealed monomeric in the mixed micellar nanoparticle with PS-block-PEO up to the therapeutic level of the drug (1-3 mM). The reduced nephrotoxicity of AmB in mixed micellar nanoparticle may be associated with the existence of the drug as monomeric form in the nanoparticle. Based on our result, formulation of AmB as mixed micellar nanoparticle with PS-block-PEO may be a promising alternative for the treatment of fungal diseases in patients who are at risk of renal dysfunction.

Key words: Mixed micellar nanoparticle, Amphotericin B, Poly styrene-block-poly ethylene oxide, Nephrotoxicity, Self-aggregation

INTRODUCTION

Amphotericin B (AmB) is a broad-spectrum antifungal agent used for the treatment of life-threatening diseases caused by various fungal strains such as *Aspergillus, Blastomyces, Cryptococcus, Coccidioides, Histoplasma*, and *Candida* species (Herbrecht *et al.*, 2005). Although AmB has been the drug of choice for those infections, the use of the drug is limited to patients in hospital-setting due to its many serious adverse effects. The major adverse effects include nephrotoxicity and infusion-related reactions such as chills, fever, hypotension, and dyspnea (Yoo *et al.*, 2006; Kleinberg, 2006). While infusion-related reactions are temporary and reversible, the nephrotoxicity associated with AmB use are oftentimes irreversible. It occurs in a dose-dependent manner and results in acute renal tubular necrosis which may progress to permanent renal failure.

Three lipid-based formulations of AmB have been introduced to clinical use in order to reduce renal toxicity of the drug: AmB lipid complex, AmB colloidal dispersion, and liposomal AmB (Mullen *et al.*, 1997; Saxena and Ghosh, 2000; Townsend *et al.*, 2001). They offer alternative formulations of AmB for the treatment of severe fungal infections in patients who are intolerant to adverse effects or whose disease is refractory to conventional formulation (Wingard *et al.*, 1999).

The advent of nanotechnology introduced new paradigm to researchers working for development of drug delivery system. This technology allows not only the improvement of solubility and stability of drugs but also the improvement of therapeutic index (Uchegbu, 2006; Kayser *et al.*, 2005). AmB is a very poorly soluble drug, and its therapeutic index is very narrow due to various adverse effects such as nephrotoxicity (Olson *et al.*, 2006). Therefore, the problems associated with the use of AmB may take

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advantage of nanotechnology to improve its solubility and nephrotoxicity profile.

In previous studies, we have synthesized decapeptide of partially benzylated poly-L-aspartic acid and reported renal toxicity reduction when AmB was administered as nanoparticular micelle with the polymer (Yoo *et al.*, 2005; Yoo *et al.*, 2006). The peptide-based nanoparticular micelle, however, may have a disadvantage associated with immunogenic problems when used as intravenous infusion to patients. Therefore, present study was undertaken to investigate whether the mixed micellar nanoparticle consisting of AmB and poly styrene-block-poly ethylene oxide (PS-block-PEO) diblock copolymer shows similar effect in terms of reducing AmB's nephrotoxicity. We have further investigated antifungal activity and self-aggregation status of the drug molecules in the nanoparticle.

MATERIALS AND METHODS

Materials

AmB (Fig. 1) was purchased from Sigma and used as obtained. Fungizone (Squibb) was kindly donated from Pharmacy Department of Yeungnam University Medical Center. *Candida albicans* (ATCC 14053) was obtained from American Type Culture Collection. Poly styrene-block-poly ethylene oxide (PS-block-PEO) was purchased from Polymer Source (Dorval, Montreal). The molecular weight of the PS block was 6,100 g/mol, and the molecular weight of the PEO block was 46,900 g/mol. All other chemicals were analytical grade and were used without further purification.

Animals

Four-week-old male Sprague-Dawley rats (140-160 g) were supplied by Orient (Seoul, Korea). Rats were separately housed in groups not exceeding three per cage and maintained under standard conditions. Food and tap water were available *ad libitum*. Room temperature was maintained at 21°C with a dark/light cycle of 12:12. Animal experiments were carried out in compliance with the appropriate institutional and national ethical guidelines for work with laboratory animals.

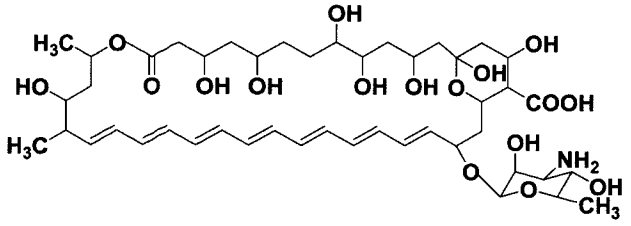

Fig. 1. Chemical structure of Amphotericin B

Preparation of mixed micellar nanoparticle of AmB and PS-block-PEO

AmB (10.0 mg) was dissolved in 4.0 ml of dimethylsulfoxide (DMSO), and the PS-block-PEO (0.7-2.0 molar equivalent to AmB) was subsequently dissolved with the aid of warming (40°C, 1 min). The solution of AmB and PS-block-PEO was diluted to an AmB level of about 25 mg/mL with isotonic PBS, pH 7.4 containing 1% DMSO. This solution was passed through high pressure homogenizer (Emulsiflex-B3, Ottawa, Canada) at 5,000 psi one time to prepare the mixed micellar nanoparticle of AmB and PS-block-PEO. The level of AmB in this solution was measured by UV/VIS spectroscopy. All the procedure was performed in the dark to avoid photodegradation of AmB.

Nephrotoxicity of mixed micellar nanoparticle of AmB and PS-block-PEO

Rats were divided into three groups and intravenously injected via penile vein with AmB in the mixed micellar nanoparticle (AmB: PS-block-PEO=1:1 molar ratio), AmB in the mixed micellar nanoparticle (AmB: PS-block-PEO= 1:2 molar ratio), and AmB micelle prepared by the same procedure without PS-block-PEO, respectively. The number of rats in each group was three, and the AmB dose was 2 mg/kg in all groups. PS-block-PEO dissolved in DMSO and diluted with isotonic PBS, pH 7.4 containing 1% DMSO (50 mg/mL) was also injected intravenously as a control group (dose=100 mg/kg). Rats were sacrificed and kidneys were removed on the eighth day. The kidneys were immersed into 2.5% glutaraldyhyde in 0.2 M phosphate-buffered solution at pH 7.3 for 3 h. The specimens were washed briefly with 0.1 M phosphate buffer, postfixed with 2% osmium tetroxide, and dehydrated through a graded ethanol series. They were then infiltrated through propylene oxide and embedded in an epoxy resin. Blocks were trimmed, and ultrathin sections (80 nm) on copper grids (150 mesh) were poststained with uranyl acetate and lead citrate before examination under transmission electron microscopy (Hitachi, H7000). Electron microscopic pictures were taken at a magnification of 2500 times (75 kV). A separate experiment was performed to measure creatinine clearance as described above with repeated dosing of 1 mg/kg for seven days instead of single dosing. Urine samples were collected by placing the rats in metabolic cages for 24 h on the seventh day. Blood samples were drawn from the abdomen aorta under anesthesia on the eighth day (immediately before sacrifice). Creatinine levels of urine and blood were measured by colorimetric method using a spectrophotometer. Creatinine clearance (C_{cr}) was calculated as follows: C_{cr} (mL/h) = urinary creatinine (mg/dl) × urinary volume (ml/h)/plasma creatinine (mg/dl).

Minimal inhibitory concentration of mixed micellar nanoparticle of AmB and PS-block-PEO

The mixed micellar nanoparticle of AmB and PS-block-PEO was diluted with isotonic PBS, pH 7.4, giving an AmB level of 10 μg/mL. In the case of conventional AmB formulation (Fungizone®), it was dissolved in 5% dextrose water. The first microwell contained 20 μL of the sample solution and 80 μL of the broth medium (RPMI 1640). The next 11 microwells were serially diluted (two-fold) with the broth medium. To each microwell, 100 μL of the inoculum which contained 5×10^3 cfu/mL of *Candida albicans* (ATCC 14053) was added, giving a total volume of 200 mL per well, and incubated at 35°C for 24 h. PS-block-PEO control and medium control were also performed simultaneously. The minimal inhibitory concentration (MIC) was defined as the minimum concentration of AmB that showed a full inhibition of the fungus in the well and examined by an inverted microscope (×40). All tests were repeated at least two times

Estimation of self-aggregation status of AmB in the mixed micellar nanoparticle

The mixed micellar nanoparticle of AmB and PS-block-PEO was diluted with isotonic PBS, pH 7.4 containing 1% DMSO and two-fold diluted with the same diluent until an AmB level of about 0.05 mg/mL was obtained. The molar absorptivity (e) at 412 nm was measured by UV/VIS spectroscopy and was plotted as a function of AmB level. AmB micelle treated by the same procedure without PS-block-PEO was also examined in the same way.

RESULTS AND DISCUSSION

AmB and PS-block-PEO were dissolved into DMSO and diluted with isotonic PBS, pH 7.4 containing 1% DMSO followed by passing through the high pressure homogenizer. Transmission electron microscopic (TEM) pictures of the resultant solution revealed nanoparticles which were mostly spherical with diameter of about 20 nm (Fig. 2). The resultant nanoparticles appeared to be mixed micelles of AmB and the PS-block-PEO because the concentrations of AmB and PS-block-PEO in the solution were far greater than their critical micelle concentrations (below 1.0 mg/mL for both, Tancrede *et al.*, 1990; Dewhurst *et al.*, 1998). There was no marked variation in the size and shape of the nanoparticles, which was consistent with characteristics of micelles.

We investigated nephrotoxicity of the mixed micellar nanoparticle of AmB and PS-block-PEO with TEM pictures of kidney of the male rats. In the groups received mixed micellar nanoparticle of AmB and PS-block-PEO (AmB: PS-block-PEO=1:1-2 molar ratio), there was no damage on the brush border of proximal tubular cells seven days after single intravenous dose of 2 mg/kg of the drug (Fig. 3A). Mitochondrial shape and inner structure did not show notable difference from those in the control group although several vacuoles in cytoplasm were found. Cell membrane of the proximal tubular cells and the size and shape of nucleus remained intact. However, in the group received AmB treated by same procedure without PS-block-PEO, there were profound damages in proximal tubular cells, and the epithelial cells were grossly swollen as well (Fig. 3B). Most mitochondria were shrunken and lost distinct inner structure and their cristae. Brush border and mitochondria were severely deformed, and there were large vacuolizations of cytoplasm. Nuclear membrane was grossly dented by the vacuoles adjacent to the nucleus, demonstrating irregular morphology. There was no damage on proximal tubular cells in the group received 100 mg/kg of PS-block-PEO dissolved in DMSO and diluted with isotonic PBS, pH 7.4 containing 1% DMSO (Fig. 3C). Damage on glomerules was not prominent in all groups tested.

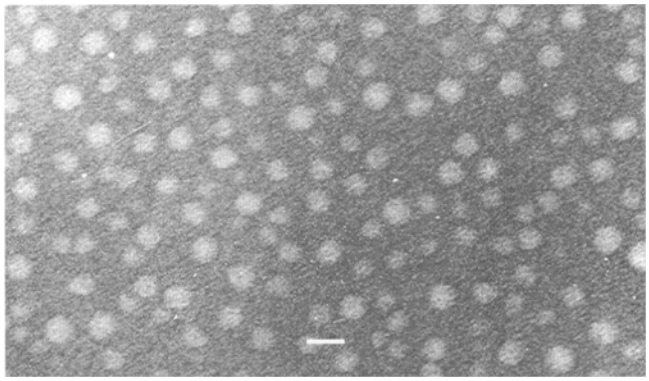

Fig. 2. Transmission electron microscopic photograph of mixed micellar nanoparticle of Amphotericin B and PS-block-PEO (molar ratio of amphotericin B to PS-block-PEO=1:2). Hitachi H7000: 50,000 times magnification at 100 kV, bar = 30 nm.

Creatinine clearances were 63.3 ± 8.2 and 59.3 ± 5.4 mL/h in rats administered with 2 mg of AmB as mixed micellar nanoparticle of AmB and PS-block-PEO with molar ratio of 1:1 and 1:2, respectively (Table I). Rats administered with AmB micelles treated by same procedure without PS-block-PEO showed a significantly decreased creatinine clearance (45.2 ± 10.5 mL/h) after seven days of repeated dosing. PS-block-PEO did not affect creatinine clearance of the rats. The creatinine clearances in the groups received mixed micellar nanoparticle represent significant reduction of nephrotoxicity compared to AmB micelles ($p<0.01$). This result indicates that the mixed micellar nanoparticle of AmB and PS-block-PEO is significantly less toxic to kidney than AmB micelles treated by same procedure without PS-block-PEO in terms of creatinine clearance. Together with the findings in TEM

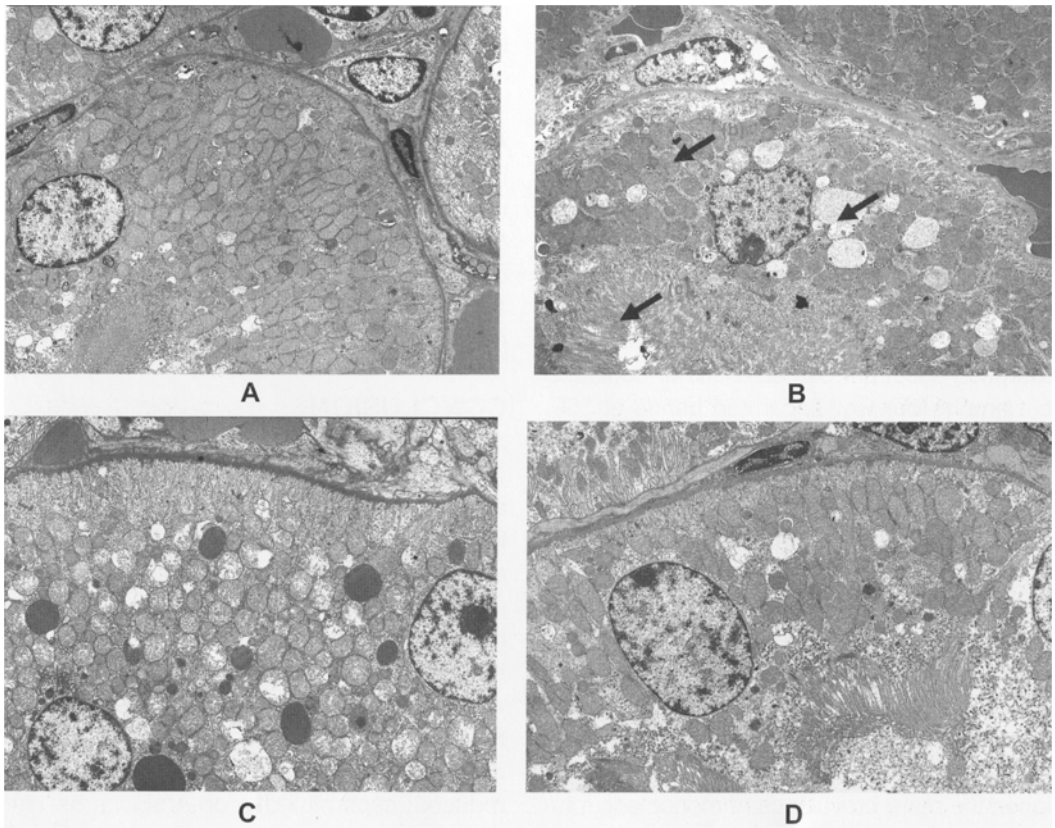

Fig. 3. Transmission electron microscopic photographs of renal tubular cells after intravenous administration of Amphotericin B. Hitachi H7000; 2500 times magnification at 75 kV. (A)=2 mg/kg of amphotericin B as mixed micellar nanoparticle of amphotericin B and PS-block-PEO with molar ratio of 1:2, (B)= 2 mg/kg of Amphotericin B treated by same procedure without PS-block-PEO, (C)=100 mg/kg of PS-block-PEO. (D)=control with isotonic PBS (pH 7.4) containing 1% dimethylsulfoxide, arrow (a)=large vacuolization, arrow (b)=damaged mitochondria, arrow (c)=damaged brush border.

Table I. Effect of mixed micellar nanoparticle of AmB and PS-block-PEO (1 mg/kg for seven days, i.v.) on creatinine clearance in rats (n=3)

	Mixed micellar nanoparticle of AmB and PS-block-PEO (molar ratio=1:1)	Mixed micellar nanoparticle of AmB and PS-block-PEO (molar ratio=1:2)	AmB micelles treated by same procedure without PS-block-PEO	PS-block-PEO (100 mg/kg)
Creatinine clearance (mL/h)	63.3±8.2**	59.3±5.4**	45.2±10.5	95.2±12.2

AmB=amphotericin B, PS-block-PEO=poly styrene-block-poly ethylene oxide, **p<0.01 compared with AmB micelles treated by same procedure without PS-block-PEO, Creatinine clearance in rats administered with isotonic PBS (pH 7.4) was 97.1±8.6 ml/h.

Table II. *In vitro* minimal inhibitory concentration (MIC) of AmB against *Candida albicans* (ATCC 14053)

	Mixed micellar nanoparticle of AmB and PS-block-PEO (molar ratio=1:1)	Mixed micellar nanoparticle of AmB and PS-block-PEO (molar ratio=1:2)	AmB micelles treated by same procedure without PS-block-PEO	AmB as Fungizone®	PS-block-PEO
MIC (μg/mL)	2.5	2.5	2.5	2.5	>2000

AmB=amphotericin B, PS-block-PEO=poly styrene-block-poly ethylene oxide

pictures, it appears that the mixed micellar nanoparticle did not harm to the tubular cells, thereby leading to less damage on glomerular filtration of the kidney.

MIC's of the mixed micellar nanoparticles of AmB and PS-block-PEO with ratio of 1:1 and 1:2 were 2.5 μg/mL (Table II) in *in vitro* using Candida albicans, one of the major fungal strains for various hospital-acquired fungal infections (Viudes *et al.*, 2002). Both MICs of conventional AmB formulation (Fungizone®) and AmB micelles treated by the same procedure without PS-block-PEO were 2.5 μg/mL. PS-block-PEO did not show antifungal activity even at the highest concentration tested (2000 μg/mL).

This result indicates that antifungal activity of the mixed micellar nanoparticle was retained while the damages on the tubular cell and creatinine clearance were significantly reduced.

We estimated the self-aggregation status of AmB molecules in the mixed micellar nanoparticle with PS-block-PEO by measurement of molar absorptivity (e) at 412 nm by UV/VIS spectroscopy. AmB molecule reveals two distinct electronic absorption spectra according to its molecular conformation (Barwicz *et al.*, 1992). When AmB is solubilized in polar organic solvent such as DMSO, it shows an absorption spectrum characteristic of heptaene chromophore, namely, four well-separated bands at 350, 368, 388, and 412 nm with the lowest peak at 350 nm and the highest peak at 412 nm. This type of spectrum is assigned to the monomeric form of AmB molecules (Barwicz *et al.*, 1992). In contrast, if AmB is solubilized with a small amount of DMSO and diluted with water, it shows a spectrum of broad single band at 328 nm showing no peak or minimal peak at 412 nm. This type of spectra is assigned to the highly self-aggregated form of the drug molecules (Rinnert *et al.*, 1977; Ernst *et al.*, 1981). Therefore, molar absorptivity (ε) of AmB at 412 nm has been suggested as a marker for self-aggregation status: the higher, the more prevalence of monomeric form (Yamashita *et al.*, 1995). Fig. 4 shows molar absorptivity of AmB at 412 nm in the mixed micellar nanoparticle with PS-block-PEO and AmB micelles without the block copolymer. When AmB was in the mixed micellar nanoparticle with with PS-block-PEO, the molar absorptivity was significantly higher than that of AmB micelles without the block copolymer up to the therapeutic level of the drug (1-3 mM). This suggests that AmB molecules in the mixed micellar nanoparticle exist as dominantly monomeric form compared to AmB without the block copolymer, especially at low level of AmB.

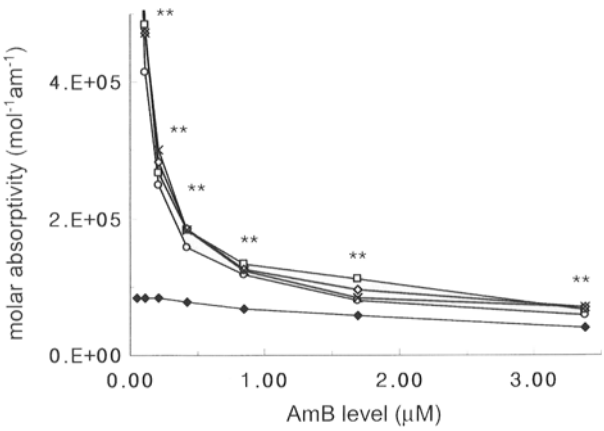

Fig. 4. Molar absorptivity of AmB in the mixed micellar nanoparticle with different molar ratio of AmB to PS-block-PEO. AmB=amphotericin B, PS-block-PEO=poly styrene-block-poly ethylene oxide, ○=1:0.7, □=1:1.0, ◇=1:1.4, ×=1:2, ◆=AmB micelle treated by same procedure without PS-block-PEO, **=p<0.01 compared to AmB micelle treated by same procedure without PS-block-PEO.

There have been many reports that self-aggregated AmB is toxic to both mammalian and fungal cell membranes while monomeric form of the drug is toxic to fungal cell membrane only (Brajtburg *et al.*, 1994; Bolard *et al.*, 1991). Therefore, the favorable result with nephrotoxicity of AmB in the mixed micellar nanoparticle appears to be associated with the existence of the drug molecules as monomeric form in the nanoparticle.

CONCLUSION

Nephrotoxicity was markedly reduced when AmB was administered to rats as mixed micellar nanoparticle in terms of transmission electron microscopy of tubular cells and creatinine clearance. Antifungal activity of AmB was not altered when the drug was in the form of mixed micellar nanoparticle compared to both the conventional formulation and AmB micelle treated by same procedure without PS-block-PEO. Self-aggregation status of AmB molecules revealed monomeric in the mixed micellar nanoparticle up to the therapeutic level of the drug. The reduced nephrotoxicity of AmB in the mixed micellar nanoparticle may be associated with the existence of the drug molecules as monomeric form in the nanoparticle. Based on our result, formulation of AmB as mixed micellar nanoparticle with PS-block-PEO may be a promising alternative for the treatment of fungal diseases in patients who are at risk of renal dysfunction.

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