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Comparison of LNS-AmB, a novel low-dose formulation of amphotericin B with lipid nano-sphere (LNS®), with commercial lipid-based formulations

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Abstract

Three lipid-based delivery systems (AmBisome®, Amphocil®, and Abelcet®) for amphotericin B (AmB) have been marketed to overcome the disadvantages associated with the clinical use of AmB. However, to show their efficacy, they need to be administered at higher doses than the conventional dosage form, Fungizone®. In this study, we compared LNS-AmB, our new low-dose therapeutic system for AmB using lipid nano-sphere (LNS®), with these commercial formulations in terms of their pharmacokinetics and efficacy. The plasma AmB levels yielded by LNS-AmB after intravenous administration to rats were much higher than those yielded by Amphocil or Abelcet, and similar to those yielded by AmBisome, but in dogs LNS-AmB yielded plasma AmB concentrations about three times higher than did AmBisome. In a carrageenin-induced pleurisy model in rats, LNS-AmB yielded AmB levels in the pleural exudate over 20 times those yielded by Amphocil or Abelcet, and similar to those yielded by AmBisome. From these pharmacokinetic results, it is clear that Amphocil and Abelcet are based on a quite distinct drug-delivery concept from LNS-AmB. In a rat model of localized candidiasis, LNS-AmB significantly inhibited the growth of *Candida albicans* in the pouch, whereas AmBisome did not, even though the AmB concentrations in the pouch were similar. This difference in antifungal activity between LNS-AmB and AmBisome was also found in vitro. That is, the antifungal activity of LNS-AmB against *C. albicans* was similar to that of Fungizone and dimethyl sulfoxide-solubilized AmB, while AmBisome showed weaker antifungal activity than did other formulations. Based on these results, the release of AmB from AmBisome was judged to be slow and slight. In a mouse model of systemic candidiasis, LNS-AmB (1.0 mg/kg) was much more effective than AmBisome (8.0 mg/kg) or Fungizone (1.0 mg/kg). These results suggest that LNS-AmB maintained the potent activity of AmB against fungal cells even though the AmB was incorporated into LNS particles. We conclude that LNS-AmB may offer an improved therapeutic profile at lower doses than Fungizone and commercial lipid-based formulations.

Keywords: Amphotericin B; Nanoemulsions; Fungizone; AmBisome; Amphocil; Abelcet

1. Introduction

Systemic fungal infections are seen with increasing frequency in immunocompromised patients (Beck-Sagué and Jarvis, 1993; Yamazaki et al., 1999).

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In spite of the recent introduction of several new antifungal agents, amphotericin B (AmB) remains one of the most effective and widely used agents for treating systemic fungal infections, as it has been for nearly 40 years (Walsh and Pizzo, 1988). Its selective activity against fungal cells is a result of its preference for binding ergosterol, the major sterol of fungal cell membranes, over cholesterol (Bolard, 1986; Kerridge, 1986). Although AmB is available as a colloidal dispersion with sodium deoxycholate (Fungizone®), its clinical use is limited by renal toxicity and other side-effects (Gallis et al., 1990).

To overcome these side-effects, three lipid-based parenteral formulations of AmB, AmBisome® (a liposomal preparation), Amphocil® (a disk-shaped complex), and Abelcet® (a ribbon-type lipid complex) have been introduced into clinical use (Table 1; Hillery, 1997). Although these industrial preparations, which differ in composition and physicochemical properties, are less toxic than Fungizone (Hiemenz and Walsh, 1998; Bekersky et al., 1999b), higher doses are needed to treat systemic fungal infections. However, since AmB itself is toxic to normal cells, and repeated administration would lead to its accumulation due to its slow elimination from the body (Atkinson and Bennett, 1978), we think that a low-dose therapeutic system for AmB is desirable to reduce its side-effects.

To develop such a system, we selected our original lipid emulsion, lipid nano-sphere (LNS®), as an injectable vehicle (Fukui et al., 2003a). LNS is composed of fine particles, 25–50 nm in diameter, which can avoid uptake by the reticuloendothelial system (RES) and can be transferred to the inflamed sites after intravenous administration (Seki et al., 1994). We have already shown that LNS incorporating AmB (LNS-AmB) yielded higher plasma concentrations of AmB than did Fungizone after intravenous administration to mice, rats, dogs, and monkeys (Fukui et al., 2003a). In addition, LNS-AmB was less toxic than Fungizone in in vitro hemolysis tests and in vivo vomiting-toxicity tests in dogs, although both formulations had nearly equal antifungal activity against fungal cells both in vitro and in vivo.

In this paper, we present the results of a comparative study of LNS-AmB and commercial formulations in which we investigated the AmB concentrations in plasma and inflamed sites and the efficacy of AmB both in vitro and in vivo, and we show that a low-dose therapeutic system with LNS-AmB has significant advantages over commercial high-dose lipid-based formulations and Fungizone.

2. Materials and methods

2.1. Materials

AmB was purchased from Dumex (Copenhagen, Denmark) and egg lecithin from QP Corporation

Table 1
Characteristics of LNS-AmB and commercial AmB formulations (from Hillery, 1997)

Product	Composition	Structure	Size	Dose[a] (mg/kg)
LNS-AmB	Soybean oil (1 g), egg lecithin (1 g), and AmB (10 mg)	Lipid emulsion	25–50 nm	?
Fungizone®	Sodium deoxycholate (41 mg) and AmB (50 mg)	Colloidal dispersion	Approximately 1 μm	0.5–1.0
AmBisome®	Hydrogenated soy phosphatidylcholine (213 mg), distearoylphosphatidylglycerol (84 mg), cholesterol (52 mg), and AmB (50 mg)	Liposome	50–100 nm	3.0
Amphocil® (ABCD)	Sodium cholesteryl sulfate (26.4 mg) and AmB (50 mg)	Disk-shaped complex	122 nm in diameter and 4 nm thick	3.0–4.0
Abelcet® (ABLC)	Dimyristoylphosphatidylcholine (68 mg), dimyristoylphosphatidylglycerol (30 mg), and AmB (100 mg)	Ribbon-type lipid complex	2–5 μm	5.0

[a] From manufacturer's instruction.

(Tokyo, Japan). All other ingredients were of pharmaceutical grade. Carrageenin was purchased from Sigma Chemical Company (St. Louis, MO) and 1-amino-4-nitronaphthalene from Aldrich (Milwaukee, WI). The solvents and reagents used in the study were of the highest commercially available grade. ^{14}C-amphotericin B (^{14}C-AmB) was produced by *Streptomyces nodosus* from [2-^{14}C] sodium acetate and used after purification by HPLC. The specific radioactivity was 1.3 MBq/mg, and the radiochemical purity, determined by thin-layer chromatography, was more than 95%.

Pharmaceutical preparations of AmB were obtained from Bristol-Myers Squibb, Princeton, NJ (Fungizone), Nexstar Pharmaceuticals, Cambridge, England (AmBisome), Sequus Pharmaceuticals, Menlo Park, CA (Amphocil), and The Liposome Co., Princeton, NJ (Abelcet), and reconstituted according to the manufacturer's instructions. The concentration of AmB in the dosage was adjusted to 0.5 mg/ml by further dilution with sterile 5% dextrose in water.

2.2. Preparation of LNS-AmB

AmB, egg lecithin and soybean oil were dissolved in chloroform–methanol (2:1, v/v). LNS-AmB contained 50 mg each of egg lecithin and soybean oil in 1 ml of the final dispersion. Organic solvent was removed under a stream of nitrogen gas and then under reduced pressure for 17 h. Complete evaporation resulted in the formation of a lipid paste, to which sterile 5% dextrose was added. The crude dispersion was emulsified with a probe-type sonicator (Sonifier model 250D, Branson Ultrasonic Corporation, Danbury, CT) in an ice-water bath for about 60 min. The final dispersion was obtained after filtration through a 0.2-μm membrane, and the final concentration of AmB in the emulsion was 0.5 mg/ml. LNS-AmB was diluted with 5% sterile dextrose if necessary.

LNS-AmB was found to be a homogeneous emulsion with mean particle diameters ranging from 25 to 50 nm by both electron microscopy (Hitachi H-7100 transmission electron microscope) and dynamic laser light scattering spectrophotometry (Otsuka DLS-7000) (Fukui et al., 2003a). The zeta potential of LNS-AmB as determined by laser dopplermicro-electrophoresis method (Malvern Zetasizer 2000) was −46 mV. LNS-AmB could be freeze-dried in the presence of certain cryoprotectants and completely rehydrated with no observed change. In addition, lyophilized LNS-AmB was stable at 25 °C for at least 2 years with no change in visual appearance, reconstitution time, pH after reconstitution, particle size, zeta potential, or AmB concentration. Based on these data, the shelf-life of lyophilized LNS-AmB is thought to be greater than 2 years at controlled room temperature.

2.3. Animals

Slc:ddY mice (4 weeks old), Sprague–Dawley rats (7 weeks old) and beagle dogs (ca. 10–12 kg) were used. For experiments on the *Candida albicans*-infected carrageenin pouch model only, 3-week-old rats were used. In all experiments, unfasted male animals were used. Mice and rats were purchased from Japan S.L.C. (Hamamatsu, Japan) and dogs from Nihon Nosan Kogyo (Yokohama, Japan). The animals were allowed to acclimate to a standard environment in the animal-care room for 1 week (mice and rats) or more than 3 weeks (dogs) before the study. All animals were allowed to take water and standard pellet chow ad libitum.

2.4. Plasma concentration profiles of AmB in rats and dogs

All AmB formulations (LNS-AmB, Fungizone, AmBisome, Amphocil, or Abelcet) were administered intravenously to rats at a dose of 1.0 mg/kg and the plasma AmB concentrations monitored as a function of time. LNS-AmB, Fungizone, or AmBisome was administered to dogs. Venous blood was collected in heparin tubes, and plasma was obtained by centrifugation at 3000 rpm for 10 min. The pharmacokinetic parameters were obtained by fitting the plasma concentrations to the two-compartment model Eq. (1) by nonlinear least-squares regression.

$$C_p = \frac{D(k_{21}-\alpha)}{V_1(\alpha-\beta)}e^{-\alpha t} + \frac{D(k_{21}-\beta)}{V_1(\beta-\alpha)}e^{-\beta t} \quad (1)$$

where C_p is the plasma concentration; D is the dose; α, β and k_{21} are the rate constants; t is time after administration; and V_1 is the distribution volume of the central compartment. The half-lives of the distribution phase ($t_{1/2}$, α) and the elimination phase ($t_{1/2}$, β) were

calculated from $\ln(2/\alpha)$ and $\ln(2/\beta)$, respectively, and the area under the plasma concentration–time curve (AUC) was calculated by a trapezoidal rule.

2.5. Carrageenin-induced pleurisy model in rats

Rat pleurisy was induced by the injection of 0.1 ml of 2% carrageenin in saline into the thorax. All AmB formulations were administered intravenously at a dose of 1.0 mg/kg 2 h after intrapleural injection of carrageenin, and AmB concentrations in the pleural exudate were monitored for 6 h after AmB administration.

2.6. Carrageenin-induced inflammation pouch model infected with C. albicans in rats

Ten milliliters of 1% carrageenin containing *C. albicans* (1.0×10^4 cells/ml) was injected subcutaneously into the back of each of four rats to induce candidiasis in the carrageenin-induced inflamed pouch. LNS-AmB, Fungizone, or AmBisome was administered intravenously at a dose of 1.0 mg/kg 3.5 h after *C. albicans* inoculation. The fluid in the pouch was collected at several time points to determine the concentration of AmB, and the fungal count in the pouch was determined 24 h after drug administration. Statistical analysis of the fungal counts was carried out according to Sheffe's test.

2.7. Determination of AmB in biological samples

The concentration of AmB in plasma, pleural exudate, and candidiasis-pouch fluid was determined by high-pressure liquid chromatography as described by Otsubo et al. (1999). Briefly, samples were deproteinized with methanol containing 1-amino-4-nitronaphthalene as an internal standard. After centrifugation, the supernatant was dried and redissolved in methanol for injection onto a reverse-phase column. The mobile phase was a mixture of acetonitrile and 10 mM sodium acetate buffer, pH 4.0 (11:17, v/v), and the flow rate was 1.0 ml/min. The eluent was monitored at 408 nm. The detection limit was 5 ng/ml and the interday and intraday coefficients of variation were 5% or less, between 5 and 400 ng/ml. In this method, free AmB and AmB retained in carrier are not determined separately.

2.8. In vitro antifungal activity

The in vitro efficacy of LNS-AmB, Fungizone, AmBisome, and AmB solubilized by dimethyl sulfoxide (DMSO) was evaluated by the inhibition of growth of *C. albicans*. Growth inhibition was measured by the decrease in optical density at 540 nm in Sabouraud dextrose broth buffered with 0.165 M 3-morpholinopropanesulfonic acid, pH 7.0, after incubation at 35 °C for 24 h.

2.9. In vivo antifungal activity

Before fungal inoculation, mice were immunosuppressed by intraperitoneal administration of cyclophosphamide at a dose of 150 mg/kg. Systemic candidiasis was then introduced by intravenous inoculation of *C. albicans* (2.5×10^4 cells/mouse). LNS-AmB, Fungizone, or AmBisome was administered intravenously at a dose of 1.0 mg/kg for LNS-AmB and Fungizone and 8.0 mg/kg for AmBisome 4 h after fungal inoculation. The mice were observed for 7 days and the in vivo systemic antifungal activity was evaluated by monitoring their survival. Statistical analysis of the survival rates was performed by a log-rank test.

2.10. Tissue distribution of AmB

LNS-AmB containing ^{14}C-AmB (LNS-^{14}C-AmB) or a colloidal dispersion containing ^{14}C-AmB prepared according to the formulation of Fungizone (DOC-^{14}C-AmB) was administered intravenously to rats at a dose of 0.5 mg/kg. After rats were killed by collecting blood from the aorta under deep ether anesthesia, tissues were removed and weighed. Plasma samples (0.1 ml) were evaporated at 50 °C under a gentle stream of nitrogen and the residues dissolved in 0.5 ml of distilled water. The radioactivity of the plasma samples was measured after the addition of 10 ml of liquid scintillator (Emulsifier Scintillator Plus, Packard Instrument Co., Downers Grove, IL). Tissue samples (about 0.2 g; liver, spleen, kidney, cerebrum, and muscle) were dried under reduced pressure, after which each sample was solubilized with 1 ml of Solvable (Packard Instrument Co.). A portion of each sample was decolorized by the addition of 0.2 ml of 30% H_2O_2. For

radioactivity measurements, 10 ml of Hionic-Fluor (Packard Instrument Co.) was added to each sample and the radioactivity measured in a Tri-Carb 3100TR liquid scintillation counter (Packard Instrument Co.) for 2 min. Counting efficiencies were corrected automatically by the external-standard-ratio method.

3. Results

3.1. Plasma concentrations of AmB in rats and dogs

In rats, the plasma AmB levels yielded by LNS-AmB were higher than those yielded by Fungizone, Amphocil, or Abelcet at all times up to 10 h (Fig. 1), and LNS-AmB and AmBisome showed similar AmB plasma profiles. In dogs, LNS-AmB yielded plasma AmB concentrations about three times higher than did AmBisome (Fig. 2). Pharmacokinetic parameters for plasma AmB in rats and dogs are summarized in Table 2.

3.2. Carrageenin-induced pleurisy model in rats

After intravenous administration of LNS-AmB, AmB concentrations in the pleural exudate were

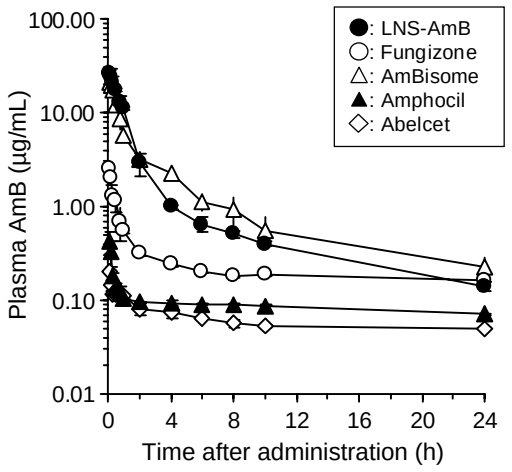

Fig. 1. Plasma concentrations of AmB after intravenous administration of LNS-AmB, Fungizone, AmBisome, Amphocil, or Abelcet at a dose of 1.0 mg/kg to rats. Each point represents the mean ± S.D. of three rats.

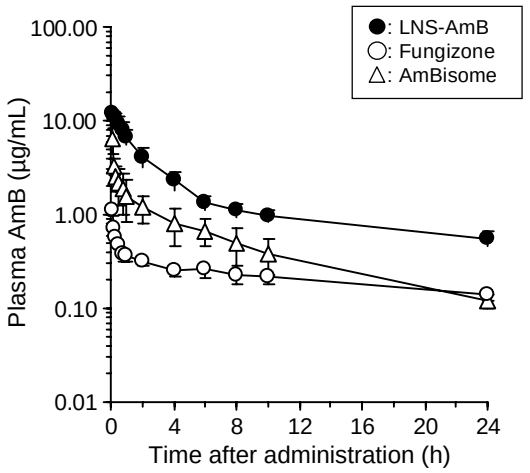

Fig. 2. Plasma concentrations of AmB after intravenous administration of LNS-AmB, Fungizone, or AmBisome at a dose of 1.0 mg/kg to dogs. Each point represents the mean ± S.D. of three dogs.

higher than those obtained after the administration of Fungizone, Amphocil, or Abelcet, and similar to those obtained after the administration of AmBisome (Fig. 3). The values of C_{max} and $AUC_{0–6\,h}$ observed for LNS-AmB were about five times those observed for Fungizone and more than 20 times those yielded by Amphocil or Abelcet (Table 3).

3.3. Carrageenin-induced inflammation pouch model infected with C. albicans in rats

In an experimental model of localized candidiasis, the AmB levels in the infected site (the pouch-fluid) after intravenous administration of LNS-AmB were about four times higher than those yielded by Fungizone and similar to those yielded by AmBisome (Fig. 4, left panel). The $AUC_{0–24\,h}$ values were 3.73 μg h/ml for LNS-AmB, 1.03 μg h/ml for Fungizone, and 3.88 μg h/ml for AmBisome.

Although both LNS-AmB and Fungizone had significantly inhibited the growth of *C. albicans* in the pouch 24 h after administration compared with the control ($P < 0.05$), there was no significant difference between AmBisome and control (Fig. 4, right panel). In addition, the inhibitory effect of LNS-AmB was significantly greater than that of Fungizone ($P < 0.05$).

Table 2
Pharmacokinetic parameters for plasma AmB after intravenous administration on LNS-AmB, Fungizone, AmBisome, Amphocil or Abelcet at a dose of 1.0 mg/kg to rats and dogs

Formulation	Rat				Dog			
	$C_{5\,min}$ (μg/ml)	($t_{1/2}$, α) (h)	($t_{1/2}$, β) (h)	$AUC_{0-24\,h}$ (μg h/ml)	$C_{5\,min}$ (μg/ml)	($t_{1/2}$, α) (h)	($t_{1/2}$, β) (h)	$AUC_{0-24\,h}$ (μg h/ml)
LNS-AmB	25.93	0.68	12.53	33.94	11.82	1.07	15.24	40.17
Fungizone	2.52	0.16	66.92	5.96	1.10	0.09	25.85	6.95
AmBisome	20.70	0.41	12.09	35.52	6.31	0.36	7.58	13.05
Amphocil	0.43	0.13	48.74	2.13		ND[a]		
Abelcet	0.20	0.12	61.55	1.48		ND		

Each value was calculated from the mean plasma concentration curve for three animals. $C_{5\,min}$ is the plasma concentration 5 min after administration, ($t_{1/2}$, α) and ($t_{1/2}$, β) are the half-lives in the distribution phase and the elimination phase, respectively, and $AUC_{0-24\,h}$ is the area under the plasma concentration–time curve up to 24 h after administration.
[a] Not determined.

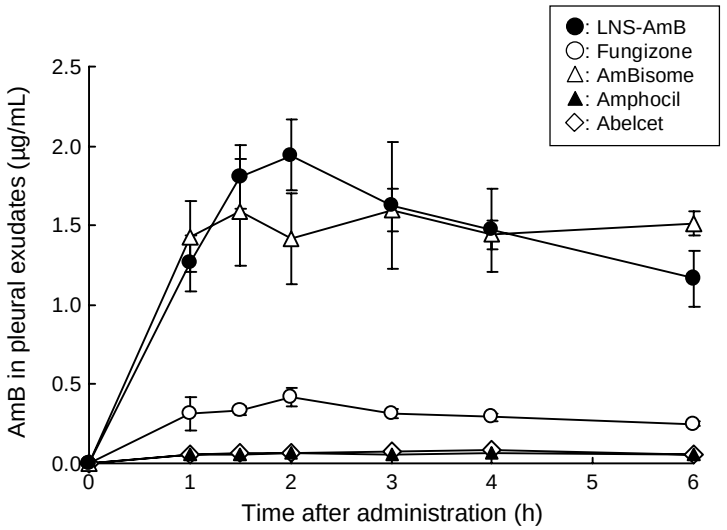

Fig. 3. Concentrations of AmB in pleural exudate after intravenous administration of LNS-AmB, Fungizone, AmBisome, Amphocil, or Abelcet at a dose of 1.0 mg/kg to rats with carrageenin-induced pleurisy. Each point represents the mean ± S.D. of four rats.

Table 3
C_{max} and AUC for AmB in pleural exudate up to 6 h after intravenous administration of LNS-AmB, Fungizone, AmBisome, Amphocil, or Abelcet at a dose of 1.0 mg/kg to rats with carrageenin-induced pleurisy

Formulation	C_{max} (μg/ml)	$AUC_{0-6\,h}$ (μg h/ml)
LNS-AmB	1.94	8.30
Fungizone	0.42	1.71
AmBisome	1.60	8.20
Amphocil	0.07	0.34
Abelcet	0.08	0.38

3.4. In vitro antifungal activity

The antifungal activity of LNS-AmB against *C. albicans* was similar to that of Fungizone and DMSO-solubilized AmB, while AmBisome showed weaker antifungal activity (Fig. 5).

3.5. In vivo antifungal activity

All untreated mice died within 2 days of fungal inoculation (Fig. 6). Although Fungizone at 1.0 mg/kg or AmBisome at 8.0 mg/kg did not significantly

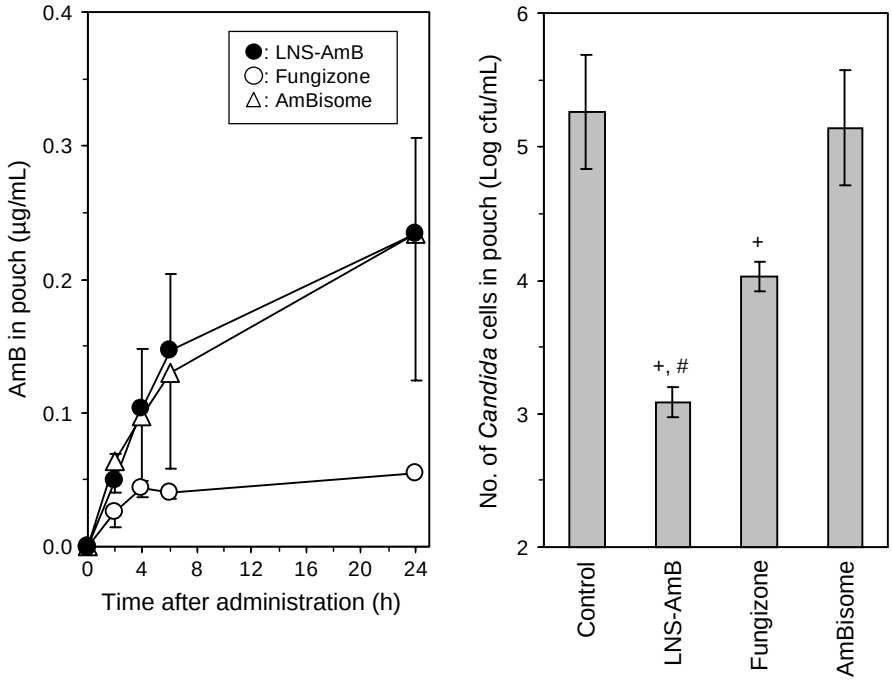

Fig. 4. Concentrations of AmB (left panel) and the fungal count (right panel) in fluid of *C. albicans*-infected carrageenin pouch after intravenous administration of LNS-AmB, Fungizone, or AmBisome at a dose of 1.0 mg/kg to rats. The fungal count was measured 24 h after drug administration. Each point represents the mean ± S.D. of four rats. (+): $P < 0.05$ compared with control; (#): $P < 0.05$ compared with Fungizone.

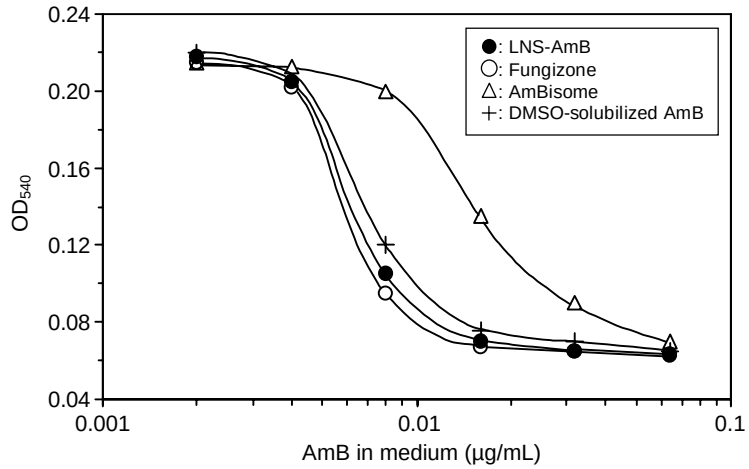

Fig. 5. Antifungal activity of LNS-AmB, Fungizone, AmBisome, and DMSO-solubilized AmB in vitro. The growth inhibition of *C. albicans* was measured by the change in optical density at 540 nm in SD-MOPS broth after a 24-h incubation at 35 °C. Results are the mean of two experiments.

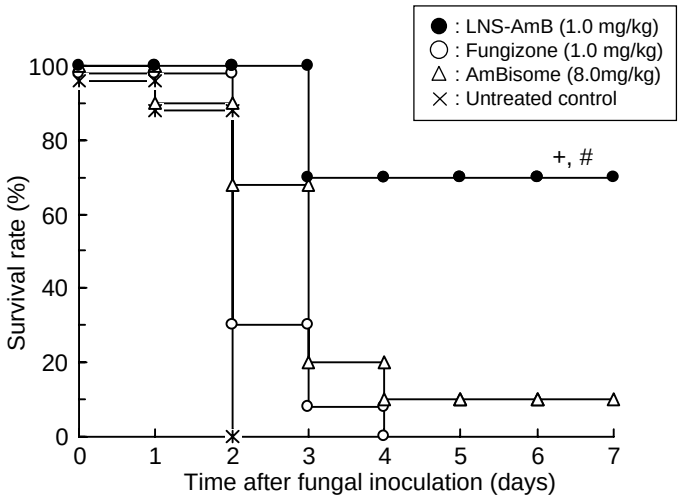

Fig. 6. Survival of mice infected with *C. albicans* and treated with LNS-AmB, Fungizone, or AmBisome. Treatment was started 4 h after fungal inoculation. (+): $P < 0.05$ compared with AmBisome; (#): $P < 0.01$ compared with Fungizone.

improve survival compared with the untreated control, LNS-AmB at 1.0 mg/kg greatly improved the survival rate and so was much more effective against systemic candidiasis than was either AmBisome ($P < 0.05$) or Fungizone ($P < 0.01$).

4. Discussion

We have shown in a previous study that LNS-AmB has the potential to become a low-dose therapeutic system for AmB (Fukui et al., 2003a,b). In the present study, we try to make clear the merits of LNS-AmB over commercial lipid-based formulations, which are themselves reported to be a significant advance in the treatment of systemic fungal infections (Hillery, 1997; Hiemenz and Walsh, 1998), by comparing their pharmacokinetics and efficacy.

In comparative studies of these delivery systems, it is important to bear in mind their physicochemical characteristics, especially the drug-to-lipid ratio, particle or vesicle size, and existence or otherwise of free drug. We found that more than 98% of the AmB and 96% of the lipids from LNS-AmB co-eluted in a single peak by size-exclusion chromatography, and that the ratio of AmB to lipid was almost constant across the peak. In addition, even if small amounts of free AmB exist in the LNS-AmB formulation, they can be removed by filtration through a 0.2 μm-membrane because the AmB is hardly soluble in water. Therefore, we concluded that free AmB hardly exists in LNS-AmB formulations and that an additional procedure for removing free AmB is not necessary for this study. Similarly, some investigators report that there is almost no free AmB in commercial lipid-based formulations (Janoff et al., 1988; Guo et al., 1991; Adler-Moore and Proffitt, 1993). The lack of free AmB in these formulations is attributed by these workers to the insolubility of AmB in water as well as its affinity to lipids.

In rats, LNS-AmB and AmBisome showed the highest plasma AmB concentrations among the formulations tested (Fig. 1; Table 2), while Amphocil and Abelcet yielded lower plasma AmB concentrations than did Fungizone. These results are consistent with previous reports (Fielding et al., 1991; Olsen et al., 1991; van Etten et al., 1995; Wang et al., 1995; Bhamra et al., 1997; Adler-Moore and Proffitt, 2002). The reason for lower AmB concentrations with Amphocil and Abelcet is probably that these formulations are mainly delivered to the RES of the liver and the spleen (Fielding et al., 1991; Olsen et al., 1991). AmBisome is also reported to be delivered to the RES, but it might have produced higher plasma AmB concentrations than did Fungizone because the extent of its distribution to the RES is lower than that of Amphocil

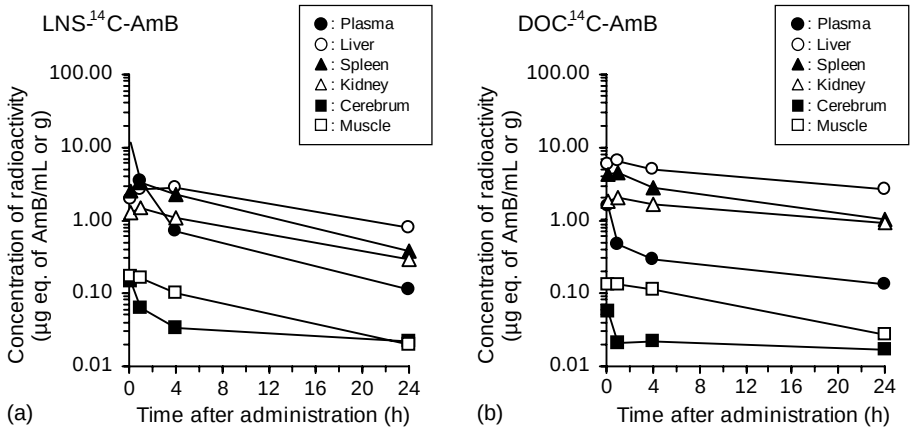

Fig. 7. Tissue concentrations of radioactivity after intravenous administration of LNS-^{14}C-AmB (a) or DOC-^{14}C-AmB (b) at a dose of 0.5 mg/kg to rats. Each point represents the mean of two rats.

and Abelcet due to its small particle size (Boswell et al., 1998a; Bekersky et al., 1999a).

In a preliminary study of the tissue distribution with ^{14}C-AmB (Fig. 7), we found that only 15% of the radioactivity administered had accumulated in the liver 5 min after intravenous administration of the LNS-^{14}C-AmB. By contrast, in the case of DOC-^{14}C-AmB, about 40% of the radioactivity administered was recovered from the liver 5 min after intravenous administration. We, therefore, consider that the long circulating lifetime of LNS-AmB is explained by its avoiding uptake by the RES due to its small particle size. Since there was not much difference in the amounts of radioactivity in the kidney between LNS-^{14}C-AmB and DOC-^{14}C-AmB at an equal dose (0.5 mg/kg), LNS-AmB is expected to deliver lower AmB concentrations to the kidney, and therefore to have reduced nephrotoxicity, if it is administered at lower doses than Fungizone. In fact, we have already reported that the nephrotoxicity of LNS-AmB is weaker than that of Fungizone in a toxicity study of daily intravenous infusion for 14 consecutive days in rats when the two formulations were administered at doses that yield almost the same AmB concentrations in the plasma (Fukui et al., 2003b).

We have also reported on the pharmacokinetics of LNS particles themselves using a radioactive tracer (Fukui et al., 2003a). The results pertaining to LNS-AmB obtained in the present study are consistent with the radioactivity observed in the plasma and tissues after intravenous administration to rats of LNS containing a radioactive tracer. In addition, we have found by size-exclusion chromatography that AmB was retained in LNS particles in the serum after intravenous administration of LNS-AmB to rats (Fukui et al., 2003b). Therefore, the pharmacokinetics of AmB as a component of LNS-AmB is thought to reflect that of the LNS particles themselves.

Based on the plasma AmB levels in rats, it is clear that the drug-delivery concept of Amphocil and Abelcet is very different from that of LNS-AmB. That is, in the case of Amphocil and Abelcet, the liver retains AmB in a form with reduced toxicity that serves as a reservoir of AmB from which AmB is slowly released (Fielding et al., 1991; Olsen et al., 1991). Therefore, we did not administer Abelcet or Amphocil in the experiment to compare the plasma AmB levels yielded by LNS-AmB and AmBisome in dogs. In this experiment, LNS-AmB yielded plasma AmB levels about three times higher than did AmBisome (Fig. 2; Table 2). Although the reason for the species difference in the plasma levels yielded by AmBisome is not clear, the difference observed in dogs between LNS-AmB and AmBisome might be explained by the difference in particle size (25–50 nm for LNS-AmB versus 50–100 nm for AmBisome). From the fact that AmBisome yields high plasma AmB concentrations in humans (Bekersky et al.,

2002), LNS-AmB would also be expected to, and this property is desirable for a low-dose therapeutic system for AmB.

To evaluate the transfer of AmB to the inflamed site after the administration of various formulations, we prepared a carrageenin-induced pleurisy model in rats. In this model, LNS-AmB and AmBisome yielded higher AmB concentrations in the pleural exudate than did Fungizone, and both Amphocil and Abelcet yielded only very low AmB concentrations (Fig. 3). Thus, the transfer of AmB to the inflamed site directly reflected the plasma AmB concentrations. In addition, LNS-AmB and AmBisome were judged to be able to permeate leaky blood vessels at inflamed sites because their particle or vesicle size was very small. With its high plasma AmB levels and passive targeting characteristics, LNS-AmB is expected to be highly efficacious in the treatment of systemic fungal infections, which are usually accompanied by inflammation. Amphocil and Abelcet were not compared in the subsequent experiments because we knew from the AmB levels in the plasma and inflamed sites that these formulations are based on quite distinct drug-delivery concepts from LNS-AmB.

We also prepared a rat carrageenin pouch model infected with *C. albicans* to evaluate the distribution of AmB to the actual infected site and the antifungal effect of LNS-AmB and AmBisome. LNS-AmB and AmBisome could deliver high levels of AmB to the infected pouch after intravenous administration (Fig. 4, left panel). However, although LNS-AmB and Fungizone significantly decreased the fungal count in the pouch compared with the control ($P < 0.05$), AmBisome did not produce a significant decrease (Fig. 4, right panel). The difference in fungal count between LNS-AmB and Fungizone clearly reflected the difference in AmB concentrations in the pouch.

In an in vitro antifungal study, AmBisome showed weaker antifungal activity than did LNS-AmB or Fungizone (Fig. 5), a result that is consistent with the findings of Carrillo-Muñoz et al. (1999). This suggests that LNS-AmB retains the potent activity of AmB against fungal cells even though the AmB is incorporated into LNS particles. Therefore, LNS-AmB in the presence of fungal cells might be expected to release AmB in sufficient amounts. By contrast, since the release of AmB from AmBisome is slow and slight, the AmB retained by AmBisome might be unable to show its strong antifungal activity even though AmBisome can deliver high levels of AmB to the infected site. We think that this is why high doses of AmBisome are required in the treatment of systemic fungal infections.

The mechanism of AmB transfer from LNS-AmB or AmBisome to fungal cells is not well understood. We have reported that LNS-AmB caused no hemolysis in vitro (Fukui et al., 2003b), and AmBisome also caused no hemolysis (Boswell et al., 1998b). Therefore, we assume that the difference in antifungal activity between LNS-AmB and AmBisome results from a selective transfer of AmB regulated by the physicochemical characteristics of the carrier and the target cells. According to our hypothesis, LNS-AmB in the presence of ergosterol-rich fungal cells readily releases AmB because the AmB has a higher affinity for ergosterol than for LNS particles. Conversely, LNS-AmB in the presence of cholesterol-rich mammalian cells retains the AmB because the AmB has a higher affinity for LNS particles than for cholesterol. On the other hand, AmBisome retains the AmB in the formulation and not readily release it even in the presence of fungal cells because the lipid components of AmBisome, such as cholesterol and distearoylphosphatidylglycerol (phase-transition temperature, about 55 °C; Szoka and Papahadjopoulos, 1980), might keep the structure of AmBisome rigid (Adler-Moore and Proffitt, 2002). In contrast, LNS-AmB is judged to be able to release AmB adequately because LNS particles are soft, being composed of only egg lecithin (phase-transition temperature, −15 to −7 °C; Szoka and Papahadjopoulos, 1980) and soybean oil.

To assess the therapeutic potential of LNS-AmB, we evaluated its in vivo antifungal activity in a *C. albicans*-infected mouse model. Several doses of LNS-AmB, Fungizone, or AmBisome, were tested and the survival rates at an optimal dose for each formulation determined. Although the survival rate for AmBisome (8.0 mg/kg) was greater than that for Fungizone (1.0 mg/kg), LNS-AmB (1.0 mg/kg) showed the best results in spite of the fact that the dosage used was eight times lower than that of AmBisome (Fig. 6). This result suggests that LNS-AmB maintained the potent activity of AmB against fungal cells in vivo.

The potential advantage of LNS-AmB lies in the high concentrations of AmB it maintains in the plasma and infected sites and its unchanged antifungal activity when administered intravenously. That is, the reduced dose is not accompanied by a decrease in therapeutic efficacy but is accompanied by a decrease in toxicity. LNS-AmB may, therefore, prove useful as a replacement for Fungizone, AmBisome, Amphocil, or Abelcet at lower doses in the treatment of systemic fungal infections. To follow up these promising results, we plan to evaluate an optimal dose of LNS-AmB in terms of efficacy and toxicity in comparison with other preparations.

References

Adler-Moore, J., Proffitt, R.T., 1993. Development, characterization, efficacy and mode of action of AmBisome®, a unilamellar liposomal formulation of amphotericin B. J. Liposome Res. 3, 429–450.

Adler-Moore, J., Proffitt, R.T., 2002. AmBisome: liposomal formulation, structure, mechanism of action and pre-clinical experience. J. Antimicrob. Chemother. 49, 21–30.

Atkinson, A.J., Bennett, J.E., 1978. Amphotericin B pharmacokinetics in humans. Antimicrob. Agents Chemother. 13, 271–276.

Beck-Sagué, C.M., Jarvis, W.R., 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. J. Infect. Dis. 167, 1247–1251.

Bekersky, I., Boswell, G.W., Hiles, R., Fielding, R.M., Buell, D., Walsh, T.J., 1999a. Safety and toxicokinetics of intravenous liposomal amphotericin B (AmBisome®) in beagle dogs. Pharm. Res. 16, 1694–1701.

Bekersky, I., Fielding, R.M., Buell, D., Lawrence, I., 1999b. Lipid-based amphotericin B formulations: from animals to man. Pharm. Sci. Technol. Today 2, 230–236.

Bekersky, I., Fielding, R.M., Dressler, D.E., Lee, J.W., Buell, D.N., Walsh, T.J., 2002. Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. Antimicrob. Agents Chemother. 46, 828–833.

Bhamra, R., Sa'ad, A., Bolcsak, L.E., Janoff, A.S., Swenson, C.E., 1997. Behavior of amphotericin B lipid complex in plasma in vitro and in the circulation of rats. Antimicrob. Agents Chemother. 41, 886–892.

Bolard, J., 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta 864, 257–304.

Boswell, G.W., Bekersky, I., Buell, D., Hiles, R., Walsh, T.J., 1998a. Toxicological profile and pharmacokinetics of a unilamellar liposomal vesicle formulation of amphotericin B in rats. Antimicrob. Agents Chemother. 42, 263–268.

Boswell, G.W., Buell, D., Bekersky, I., 1998b. AmBisome (liposomal amphotericin B): a comparative review. J. Clin. Pharmacol. 38, 583–592.

Carrillo-Muñoz, A.J., Quindõs, G., Tur, C., Ruesga, M.T., Miranda, Y., del Valle, O., Cossum, P.A., Wallace, T.L., 1999. In-vitro antifungal activity of liposomal nystatin in comparison with nystatin, amphotericin B cholesteryl sulphate, liposomal amphotericin B, amphotericin B lipid complex, amphotericin B desoxycholate, fluconazole and itraconazole. J. Antimicrob. Chemother. 44, 397–401.

Fielding, R.M., Smith, P.C., Wang, L.H., Porter, J., Guo, L.S.S., 1991. Comparative pharmacokinetics of amphotericin B after administration of a novel colloidal delivery system, ABCD, and a conventional formulation to rats. Antimicrob. Agents Chemother. 35, 1208–1213.

Fukui, H., Koike, T., Saheki, A., Sonoke, S., Seki, J., 2003a. A novel delivery system for amphotericin B with lipid nano-sphere (LNS®). Int. J. Pharm. 265, 37–45.

Fukui, H., Koike, T., Saheki, A., Sonoke, S., Tomli, Y., Seki, J., 2003b. Evaluation of the efficacy and toxicity of amphotericin B incorporated in lipid nano-sphere (LNS®). Int. J. Pharm. 263, 51–60.

Gallis, H.A., Drew, R.H., Pickard, W.W., 1990. Amphotericin B: 30 years of clinical experience. Rev. Infect. Dis. 12, 308–329.

Guo, L.S.S., Fielding, R.M., Lasic, D.D., Hamilton, R.L., Mufson, D., 1991. Novel antifungal drug delivery: stable amphotericin B-cholesteryl sulfate discs. Int. J. Pharm. 75, 45–54.

Hiemenz, J.W., Walsh, T.J., 1998. Lipid formulations of amphotericin B. J. Liposome Res. 8, 443–467.

Hillery, A.M., 1997. Supramolecular lipidic drug delivery systems: from laboratory to clinic. A review of the recently introduced commercial liposomal and lipid-based formulations of amphotericin B. Adv. Drug Deliv. Rev. 24, 345–363.

Janoff, A.S., Boni, L.T., Popescu, M.C., Minchey, S.R., Cullis, P.R., Madden, T.D., Taraschi, T., Gruner, S.M., Shyamsunder, E., Tate, M.W., Mendelsohn, R., Bonner, D., 1988. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc. Natl. Acad. Sci. U.S.A. 85, 6122–6126.

Kerridge, D., 1986. Mode of action of clinically important antifungal drugs. Adv. Microb. Physiol. 27, 1–72.

Olsen, S.J., Swerdel, M.R., Blue, B., Clark, J.M., Bonner, D.P., 1991. Tissue distribution of amphotericin B lipid complex in laboratory animals. J. Pharm. Pharmacol. 43, 831–835.

Otsubo, T., Maesaki, S., Hossain, M.A., Yamamoto, Y., Tomono, K., Tashiro, T., Seki, J., Tomii, Y., Sonoke, S., Kohno, S., 1999. In vitro and in vivo activities of NS-718, a new lipid nanosphere incorporating amphotericin B, against *Aspergillus fumigatus*. Antimicrob. Agents Chemother. 43, 471–475.

Seki, J., Sasaki, H., Doi, M., Yoshikawa, H., Takahashi, Y., Yamane, S., Sonoke, S., Yamamoto, H., Hirose, M., Ezure, Y., Ando, T., Ushimaru, K., Sugiyama, M., 1994. Lipid nano-sphere (LNS), a protein-free analogue of lipoproteins, as a novel drug carrier for parenteral administration IV. J. Control. Release 28, 352–353.

Szoka, F., Papahadjopoulos, D., 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu. Rev. Biophys. Bioeng. 9, 467–508.

van Etten, E.W.M., Otte-Lambillion, M., ten Kate, M.T., Bakker-Woudenberg, I.A.J.M., 1995. Biodistribution of liposomal amphotericin B (AmBisome) and amphotericin B-desoxycholate (Fungizone) in uninfected immunocompetent mice and leucopenic mice infected with *Candida albicans*. J. Antimicrob. Chemother. 35, 509–519.

Walsh, T.J., Pizzo, A., 1988. Treatment of systemic fungal infections: recent progress and current problems. Eur. J. Clin. Microbiol. Infect. Dis. 7, 460–475.

Wang, L.H., Fielding, R.M., Smith, P.C., Guo, L.S.S., 1995. Comparative tissue distribution and elimination of amphotericin B colloidal dispersion (Amphocil®) and Fungizone® after repeated dosing in rats. Pharm. Res. 12, 275–283.

Yamazaki, T., Kume, H., Murase, S., Yamashita, E., Arisawa, M., 1999. Epidemiology of visceral mycoses: analysis of data in annual of the pathological autopsy cases in Japan. J. Clin. Microbiol. 37, 1732–1738.