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A novel delivery system for amphotericin B with lipid nano-sphere (LNS®)

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Abstract

A low-dose therapeutic system with a lipid emulsion for amphotericin B (AmB), a potent antifungal drug, was studied. Lipid nano-sphere (LNS®), a small-particle lipid emulsion, was taken up by the liver to a lesser extent than was a conventional lipid emulsion. As a result, LNS yielded higher plasma concentrations of a radiochemical tracer than did the conventional lipid emulsion. LNS was therefore judged to be a suitable carrier for a low-dose therapeutic system for AmB, and LNS incorporating AmB (LNS-AmB) was prepared. LNS-AmB was found to be a homogeneous emulsion with mean particle diameters ranging from 25 to 50 nm. LNS-AmB yielded higher plasma concentrations of AmB than did Fungizone®, a conventional intravenous dosage form of AmB, after intravenous administration to mice, rats, dogs, and monkeys. This difference between LNS-AmB and Fungizone was also observed for constant intravenous infusion. In contrast to Fungizone, LNS-AmB showed a linear relationship between dose and AUC. These pharmacokinetic characteristics of LNS-AmB make it a suitable candidate for an effective low-dose therapeutic system for AmB.

Keywords: Amphotericin B; Nanoemulsions; Lipid emulsion; LNS

1. Introduction

The most effective clinically available treatment for systemic fungal infections is Fungizone®, an intravenously administered colloidal dispersion of amphotericin B (AmB) with sodium deoxycholate. However, the clinical efficacy of Fungizone is limited both by severe and acute toxic side effects, such as fever, chills, hemolysis, and vomiting, and by symptoms of nephrotoxicity, which develop after several weeks of therapy (Gallis et al., 1990). Several strategies have been developed to overcome these disadvantages of AmB. As a result, three new delivery systems for AmB (AmBisome®, Amphocil®, and Abelcet®) with reduced side effects are now on the market (Table 1; Hillery, 1997).

These new dosage forms are reported to have excellent safety and efficacy (Szoka and Tang, 1993; Hiemenz and Walsh, 1998). However, comparable efficacy can be achieved only when they are administered at higher doses than Fungizone. It is desirable to lower the therapeutic dose of AmB because its elimination from the body is very slow (Atkinson and Bennett, 1978; Daneshmend and Warnock, 1983) and repeated administration would lead to its accumulation. So we set out to develop a low-dose delivery system for AmB to reduce its side effects while maintaining its activity against fungal cells.

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

LNS-AmB	Small lipid emulsion composed of soybean oil and egg lecithin (25–50 nm)
Fungizone®	Colloidal dispersion with sodium deoxycholate (approximately 1 μm)
AmBisome®	Liposome composed of hydrogenated soy phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol (50–100 nm)
Amphocil® (ABCD)	Uniform disk-shaped complex with sodium cholesteryl sulfate (122 nm in diameter and 4 nm thick)
Abelcet® (ABLC)	Ribbon-type lipid complex composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (2–5 μm)

AmB is an amphiphilic molecule with a high affinity for membrane sterols (Kerridge, 1986; Brajtburg and Bolard, 1996). Lipid emulsions have been used clinically for parenteral nutrition and as carriers for lipophilic drugs for a long time (Collins-Gold et al., 1990; Mizushima, 1996). However, it is thought that lipid microsphere (LM), a conventional lipid emulsion, cannot penetrate infected sites because of its large particle diameter (about 200 nm). We have previously reported that lipid nano-sphere (LNS®), a lipid emulsion of small particles, is an excellent carrier for lipophilic drugs (Seki et al., 1994). For example, LNS confers on some lipophilic drugs a prolonged plasma half-life, increased plasma concentrations, low uptake by the reticuloendothelial system (RES), and good distribution to inflamed sites through blood vessels with increased permeability. In this study, we compare the pharmacokinetic characteristics of LNS and LM and evaluate the potential of LNS incorporating AmB (LNS-AmB) to become a low-dose delivery system for AmB.

2. Materials and methods

2.1. Materials

AmB was purchased from Dumex (Copenhagen, Denmark), ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) from NEN (Wilmington, DE, USA), egg lecithin from QP Corporation (Tokyo, Japan), and 1-amino-4-nitronaphthalene from Aldrich (Milwaukee, WI, USA). All other ingredients were of pharmaceutical grade. The solvents and reagents used were of the highest commercially available grade and were used without further purification.

Fungizone (50 mg AmB per vial; Bristol-Myers Squibb, Princeton, NJ, USA) was reconstituted with 10 ml of water for injection and diluted 1:9 (v/v) with sterile 5% dextrose in water to a concentration of 0.5 mg/ml.

2.2. Preparation of lipid emulsions

The emulsions LNS and LM, containing AmB or ^{3}H-CHE, were prepared as follows. Egg lecithin, soybean oil, and AmB or ^{3}H-CHE, were dissolved in chloroform–methanol (2:1, v/v). LNS contained 50 mg each of egg lecithin and soybean oil in 1 ml of the final dispersion, while LM contained 12 mg of egg lecithin and 100 mg of 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, USA) in an ice-water bath for about 60 min (LNS) or 20 min (LM). The final dispersion for the study was obtained after filtration through a 0.2-μm membrane (LNS) or a 0.45-μm membrane (LM), the final concentration of AmB in the emulsions was 0.5 mg/ml, and the final radiochemical concentration of ^{3}H-CHE was 37 kBq/ml.

2.3. Morphology of emulsion

The particle size range was determined with a dynamic laser light scattering spectrophotometer (DLS-7000; Otsuka Electronics, Osaka, Japan) after dilution of the emulsion with distilled water. For transmission electron microscopy, the samples were placed on a specimen mesh coated with collodion film, negatively stained with 3% sodium phosphotungstate, pH 7.0, and observed with a Hitachi H-7100 transmission electron microscope.

2.4. Animals

Slc:ddY mice (7 weeks old), Sprague–Dawley rats (7 weeks old), beagle dogs (9–12 kg), and cynomolgus monkeys (4–5 kg) were used. Unfasted male animals were used in all experiments. Mice, rats, and monkeys were purchased from Japan S.L.C. (Hamamatsu, Japan), and dogs were purchased from Nihon Nosan Kogyo (Yokohama, Japan). The animals were allowed to acclimate to an environment maintained at 21–25 °C and 45–65% humidity in the animal-care room for 1 week (mice and rats), 3 weeks (dogs), or 2 months (monkeys). All animals were allowed to take water and standard pellet chow ad libitum.

2.5. Measurement of radioactivity in plasma and tissues

LNS or LM containing ^{3}H-CHE was administered intravenously to rats at a dose of 74 kBq/kg. Venous blood was collected in heparin tubes, and plasma was obtained by centrifugation at 3000 rpm for 10 min. 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, USA).

After rats were killed by collecting blood from the aorta under deep ether anesthesia, tissues were removed and weighed. Tissue samples (about 0.2 g) or suspensions of liver cells (0.5 ml) prepared as described below were dried under reduced pressure, after which each sample was solubilized with 1 ml of Solvable (Packard). 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) was added to each sample and the radioactivity measured in a Tri-Carb 3100TR liquid scintillation counter (Packard) for 2 min. Counting efficiencies were corrected automatically by the external standard ratio method.

2.6. Isolation of liver cells

Pure and intact parenchymal cells and non-parenchymal cells from rat liver were isolated as described by Watanabe et al. (1993) with minor modifications. First, the liver was pre-perfused with perfusion buffer (0.50 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 136.9 mM NaCl, 5.4 mM KCl, 10.0 mM HEPES, 0.50 mM EGTA, 4.2 mM $NaHCO_3$, and 5.0 mM glucose, pH 7.3) for approximately 10 min. Then, the liver was perfused with a buffer containing 0.05% collagenase and 0.01% trypsin inhibitor (pH 7.5) for approximately 20 min. Perfusion was performed at 37 °C at a flow rate of 20 ml/min. The liver was then removed from the rat and minced with scissors, if necessary. A whole-liver suspension was made by carefully pipetting the tissue in Dulbecco's Modified Eagle Medium (DMEM) and filtering it through nylon gauze. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant discarded. The pellet was redispersed into DMEM and centrifuged at 500 rpm for 1 min to sediment the parenchymal cells. This step was repeated two more times. Non-parenchymal cells remained in the supernatant and parenchymal cells were found in the final pellet. All centrifugation steps were done at 5 °C. At least 90% of the cells obtained were viable according to the trypan blue exclusion test, and cross-contamination between parenchymal cells and non-parenchymal cells was less than 5% as determined by light microscopy.

2.7. Plasma concentration profiles of AmB

LNS-AmB or Fungizone was administered intravenously to mice, rats, dogs, and monkeys at a dose of 1.0 mg/kg. For constant intravenous infusion, LNS-AmB or Fungizone was administered at a dose of 0.25, 0.5, or 1.0 mg/kg over a period of 4 h. Plasma samples were prepared as described above.

2.8. Measurement of AmB concentration in plasma

The concentration of AmB in plasma was determined by high-pressure liquid chromatography as described by Otsubo et al. (1999). Plasma (0.1 ml) was deproteinized by vortexing it with methanol (1.0 ml) containing 1.0 μg internal standard 1-amino-4-nitronaphthalene/ml. After centrifugation at 3000 rpm for 10 min, the supernatant was decanted, dried down under reduced pressure, redissolved in 0.2 ml of methanol, and injected onto a reverse-phase column (L-column, 4.6 mm × 150 mm; Chemicals Inspection

and Testing Institute, Tokyo, Japan). The mobile phase was a mixture of acetonitrile and 10 mM sodium acetate buffer, pH 4.0 (11:17, v/v), the flow rate was 1.0 ml/min, and 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. If the concentration of AmB was over 400 ng/ml, the sample was diluted with blank plasma.

2.9. Calculation of pharmacokinetic parameters

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 in 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.

3. Results

3.1. Comparative pharmacokinetic study of LNS and LM

^{3}H-CHE yielded higher plasma radioactivity levels when incorporated into LNS than when incorporated into LM (Fig. 1). The $AUC_{0-4\,h}$ was 15.93% of dose h/ml for LNS and 2.32% of dose h/ml for LM. In the distribution phase, LNS showed a half-life of 0.82 h and LM a half-life 0.15 h.

After intravenous administration of LM containing ^{3}H-CHE, 70–80% of the radioactivity had accumulated in the liver and spleen by 0.5 h, with only 10% left in the blood, whereas after administration of LNS containing ^{3}H-CHE more than 50% of the radioactivity remained in the blood at this time (Fig. 2). LM delivered larger amounts of radioactivity to the non-parenchymal cells than to the parenchymal cells in the liver, while LNS delivered almost equal amounts to both cell types (Fig. 3).

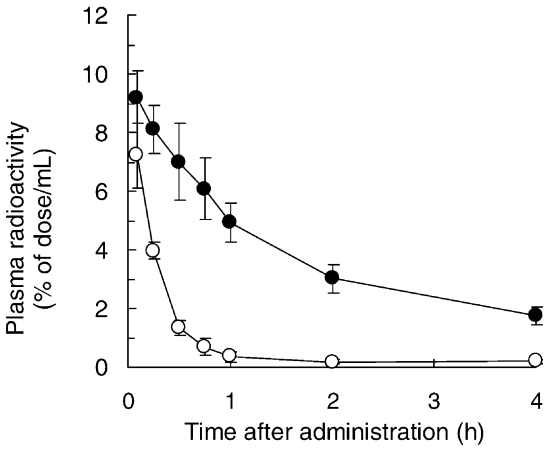

Fig. 1. Plasma radioactivity after intravenous administration to rats of lipid emulsions incorporating ^{3}H-CHE. Each point represents the mean ± S.D. of three rats. Closed circles, LNS; open circles, LM.

3.2. Morphology of emulsion

Dynamic laser light scattering and electron microscopy showed that LNS-AmB was a homogeneous emulsion with mean particle diameters ranging from 25 to 50 nm (Fig. 4 and Plate 1). Similar results were obtained for AmB-free LNS. The mean particle diameter for AmB-free LM was about 200 nm.

3.3. Plasma concentration of AmB

When LNS-AmB or Fungizone was intravenously administered to mice, rats, dogs, and monkeys at a dose of 1.0 mg/kg, LNS-AmB yielded higher plasma AmB concentrations in all species than did Fungizone (Fig. 5). The values of $AUC_{0-24\,h}$ observed for LNS-AmB were two to six times higher than those yielded by Fungizone (Table 2). Both LNS-AmB and Fungizone showed the highest plasma AmB levels in monkeys, while the ratios of $AUC_{0-24\,h}$ for LNS-AmB to $AUC_{0-24\,h}$ for Fungizone were in the rank order dogs = rats > monkeys > mice. The corresponding ratios for $AUC_{0-4\,h}$ showed the same rank order but were approximately twofold greater.

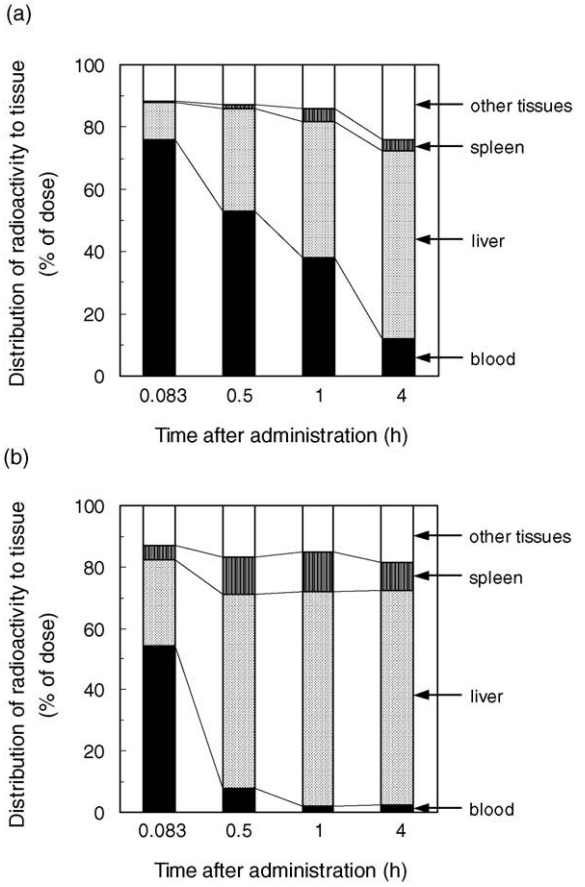

Fig. 2. Tissue distribution of radioactivity after intravenous administration to rats of lipid emulsions incorporating ^{3}H-CHE. Values are the mean of four rats. (a) LNS; (b) LM.

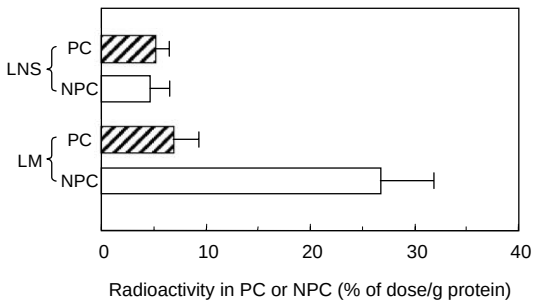

Fig. 3. Uptake of radioactivity by liver parenchymal cells (PC) and non-parenchymal cells (NPC) 15 min after intravenous administration to rats of lipid emulsions incorporating ^{3}H-CHE.

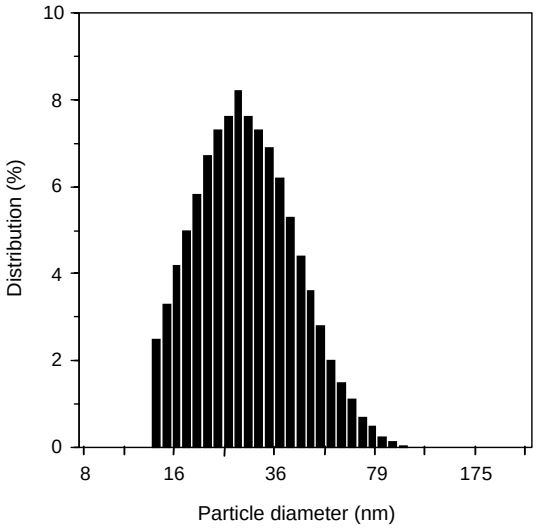

Fig. 4. Distribution of particle diameters of LNS-AmB measured by dynamic laser light scattering spectrophotometry.

When LNS-AmB or Fungizone was administered to dogs by constant intravenous infusion at a dose of 1.0 mg/kg, the plasma AmB concentrations were higher for LNS-AmB (Fig. 6). The ratio of $AUC_{0–24\,h}$ for LNS-AmB to $AUC_{0–24\,h}$ for Fungizone after constant infusion was 5.6, similar to the ratio observed after bolus injection. $AUC_{0–24\,h}$ for LNS-AmB

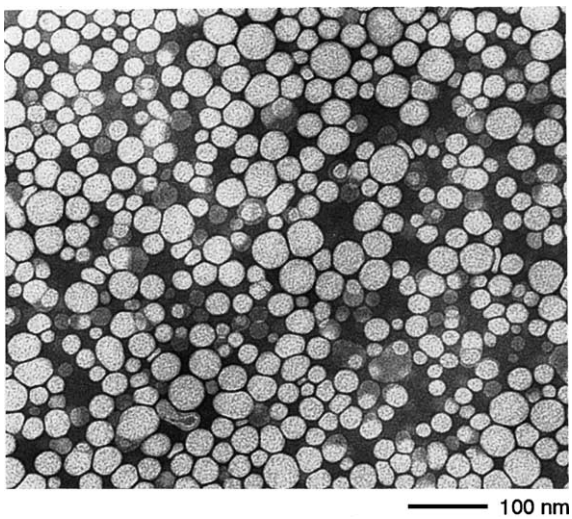

Plate 1. Electron micrograph of negatively stained LNS-AmB.

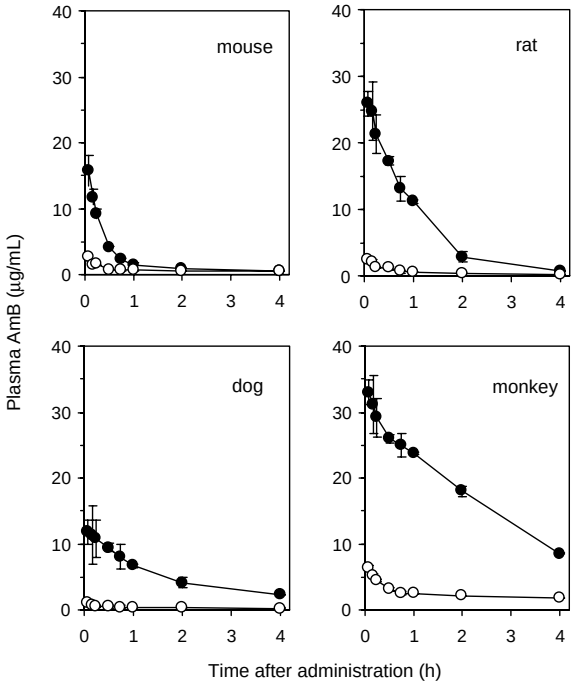

Fig. 5. Plasma concentrations of AmB after intravenous administration of LNS-AmB or Fungizone at a dose of 1.0 mg/kg to mice, rats, dogs, and monkeys. Each point represents the mean ± S.D. of three animals. Closed circles, LNS-AmB; open circles, Fungizone.

increased linearly in proportion to the dose, whereas $AUC_{0-24\,h}$ for Fungizone did not (Fig. 7). $AUC_{0-24\,h}$ for 1.0 mg/kg Fungizone (5.03 μg h/ml) was approximately equal to $AUC_{0-24\,h}$ for 0.25 mg/kg LNS-AmB (6.47 μg h/ml).

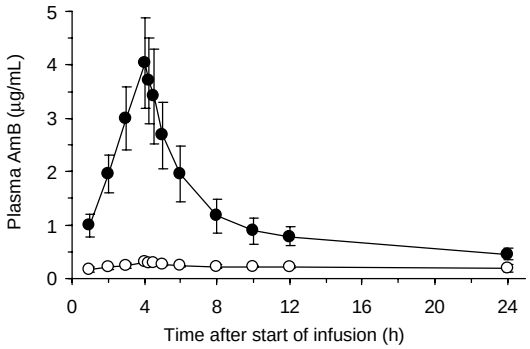

Fig. 6. Plasma concentrations of AmB after constant intravenous infusion of LNS-AmB or Fungizone into dogs for 4 h at a dose of 1.0 mg/kg. Each point represents the mean ± S.D. of three dogs. Closed circles, LNS-AmB; open circles, Fungizone.

4. Discussion

The purpose of our research was to establish an effective low-dose therapeutic system for AmB. Among drug carriers with a long circulating lifetime in the blood, liposome preparations and lipid emulsions are candidates for intravenously injectable carriers that can be produced on an industrial scale. Though liposomes can be useful for either hydrophilic or lipophilic drugs, their capacity for lipophilic drugs is generally less than for hydrophilic drugs (Barenholz and Cohen, 1995). This characteristic of liposomes would appear to make them unsuitable as a carrier for a low-dose therapeutic system for AmB, which is practically insoluble in water. Therefore, we decided to use a lipid emulsion as the carrier, and in this paper we evaluate

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

Animal	$C_{5\,min}$ (μg/ml)		$t_{1/2}$, α (h)		$t_{1/2}$, β (h)		$AUC_{0-24\,h}$ (μg h/ml)		
	LNS-AmB	Fungizone	LNS-AmB	Fungizone	LNS-AmB	Fungizone	LNS-AmB	Fungizone	Ratio
Mouse	15.71	2.72	0.20	0.17	21.43	15.17	15.40	8.31	1.9
Rat	25.93	2.52	0.68	0.16	12.53	66.92	33.94	5.96	5.7
Dog	11.82	1.10	1.07	0.09	15.24	25.85	40.17	6.95	5.8
Monkey	32.96	6.32	0.68	0.20	8.29	24.12	144.30	36.86	3.9

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, calculated by a trapezoidal rule.

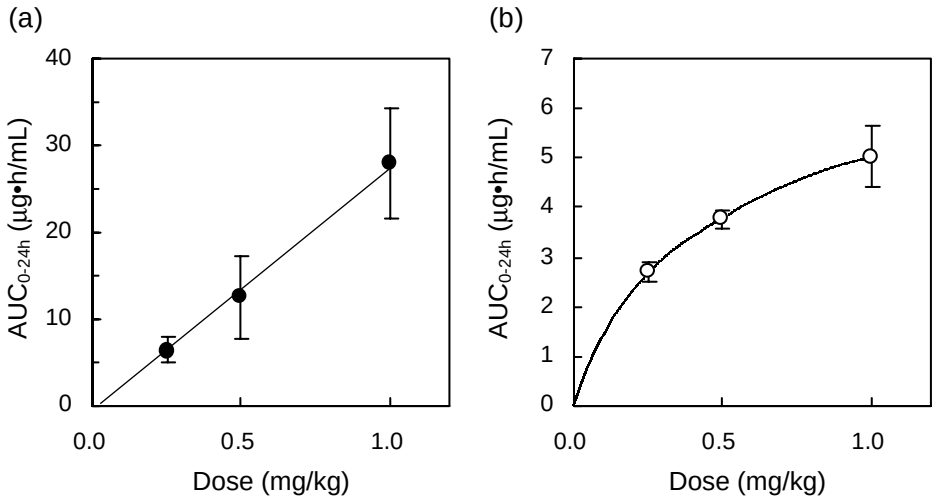

Fig. 7. Relationship between dose and $AUC_{0-24\,h}$ during and after constant intravenous infusion of (a) LNS-AmB or (b) Fungizone into dogs for 4 h at a dose of 0.25, 0.5, and 1.0 mg/kg. Each point represents the mean ± S.D. of three dogs. Regression line for LNS-AmB: $AUC_{0-24\,h} = 28.954 \times \text{dose} - 1.260$.

the potential of a novel delivery system for AmB with LNS.

First, we compared a conventional lipid emulsion (LM) and a small-particle lipid emulsion (LNS) to determine which would be the more suitable for this research. In our previous study on dexamethasone palmitate (Seki et al., 1994), LNS remained in circulation for longer than LM. On the other hand, we have also found that when LNS and LM incorporated certain lipophilic fluorescent compounds and intravenously administered to rats, the plasma concentration profiles were not very different (Fukui et al., 1994). We think that these compounds are transferred to albumin or lipoproteins immediately after intravenous administration of the emulsion formulations. In addition, since there are no comparative pharmacokinetic studies on LNS and LM themselves, we first evaluated the circulation characteristics of the emulsions labeled with ^{3}H-CHE. After intravenous administration to rats, LNS yielded an approximately sevenfold higher AUC for plasma radioactivity than did LM. In addition, distribution studies suggested that LM particles were more easily captured by the RES of, for example, the liver and spleen than were LNS particles. This difference could be explained by the relatively low distribution of LNS particles to non-parenchymal cells, such as Kupffer cells and endothelial cells on the vascular capillary bed (see Fig. 3). Non-parenchymal cells actively participate in the uptake of foreign colloidal and particulate matter (Knook et al., 1977), and LM might be recognized as foreign matter. However, LNS could avoid uptake because the particles were too small to be recognized by non-parenchymal cells, and in this respect it resembled an endogenous lipoprotein.

We have already found that the difference in plasma profile between LNS and LM can be explained by the difference in the binding affinity of apolipoprotein E (Apo E) and apolipoprotein C-II (Apo C-II) to the emulsion particles (Seki et al., in preparation). Immediately after injection of LM, Apo E and Apo C-II bind to the surface of the particles, and the LM particles then disappear smoothly from the bloodstream due to hydrolysis by lipoprotein lipase (LPL) and subsequent uptake into the liver mediated by the Apo E receptor. Apo E and Apo C-II showed a lower affinity for LNS particles compared with LM, and the higher plasma concentrations of ^{3}H-CHE obtained with LNS probably resulted from a slower hydrolysis by LPL and a consequent slower uptake by the liver. Since longer circulation in the bloodstream could be achieved by reducing the particle size of the emulsion, we decided to use LNS as a carrier to establish a low-dose therapeutic system for AmB.

The fineness of the LNS-AmB particles was demonstrated by both electron microscopy and dynamic laser light scattering spectrophotometry. Because LNS-AmB can be filtered through a 0.2-μm membrane for sterilization, it is suitable for injection. In addition, LNS-AmB was judged to be as stable as three lipid-based commercial formulations (AmBisome, Amphocil, and Abelcet) because it could be freeze-dried in the presence of certain cryoprotectants and completely rehydrated with no observed changes.

To study the circulation properties of LNS-AmB in the blood, LNS-AmB or Fungizone was intravenously administered to mice, rats, dogs, and monkeys at a dose of 1.0 mg/kg. Higher plasma AmB concentrations were observed for LNS-AmB than for Fungizone, presumably because LNS particles could circulate longer in the blood by avoiding hydrolysis by LPL and uptake by the RES. In a separate experiment, we found that the minimum inhibitory concentration (MIC) against *Candida albicans* is 0.125 μg/ml for LNS-AmB and 0.25 μg/ml for Fungizone. Therefore, it is easy for LNS-AmB to achieve this MIC level in the plasma even if LNS-AmB is administered at a lower dose than Fungizone. We have already carried out a comparative study on the plasma AmB concentrations of other lipid-based formulations (Fukui et al., submitted). We found that the plasma AmB levels yielded by Amphocil and Abelcet in rats were about three times lower than those yielded by Fungizone, and that AmBisome and LNS-AmB showed similar AmB plasma profiles. Although AmBisome yielded higher plasma concentrations than did Fungizone, we believe that to treat fungal infections it should be administered at about three times the dose required for Fungizone as recommended by the manufacturer, due to the slow release of AmB from AmBisome.

LNS-AmB and Fungizone showed similar plasma profiles in the terminal phase. The disappearance of AmB itself from the circulation was extremely slow, and the terminal half-life of AmB between 72 and 168 h after administration to monkeys was 75 h for LNS-AmB and 89 h for Fungizone. In addition, the ratio of plasma AmB concentration for LNS-AmB to plasma AmB concentration for Fungizone in various species was greater than twofold for several hours, although there were species differences (see Fig. 8). Hence, we conclude that the effect of LNS in maintaining high plasma levels of AmB continued for several hours after intravenous administration.

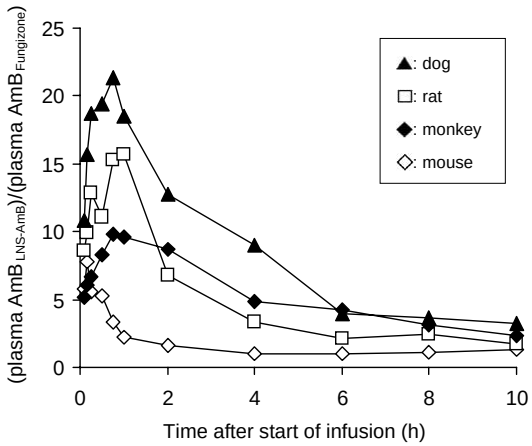

Fig. 8. Ratios of plasma AmB concentration for LNS-AmB to plasma AmB concentration for Fungizone after intravenous administration to mice, rats, dogs, and monkeys. Each ratio was calculated from the data shown in Fig. 5. Open diamond, mouse; open square, rat; closed triangle, dog; closed diamond, monkey.

In clinical use, Fungizone is infused for more than 4 h. When we compared the plasma levels of AmB after constant infusion of LNS-AmB or Fungizone to dogs for 4 h, we obtained essentially the same results as for bolus injection. Therefore, the long circulating lifetime of LNS particles was not affected by the method of administration, a property that has merit in clinical use. In contrast to AUC for LNS-AmB, AUC for Fungizone did not increase in proportion to the dose. Since Fungizone yields a precipitate when reconstituted in saline, higher doses would be expected to yield larger colloidal particles in the bloodstream, and these would be more easily captured by the RES.

In this study, we have developed a potential low-dose delivery system for AmB as an alternative to the high-dose delivery system commercially available. This low-dose system is based on the carrier LNS, which has a long circulating lifetime. LNS incorporating AmB (LNS-AmB) could be sterilized by filtration and could maintain high plasma levels of AmB regardless of the schedule of administration. We have already carried out comparative studies on the efficacy and toxicity of LNS-AmB compared with other commercially available AmB formulations, and have obtained encouraging results (Fukui et al.,

submitted). We believe that these favorable characteristics of LNS-AmB will allow the development of a low-dose AmB therapy for systemic fungal infections.

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