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Research Article

Development of Amphotericin B-Loaded Cubosomes Through the SolEmuls Technology for Enhancing the Oral Bioavailability

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Abstract. The oral administration of amphotericin B (AmB) has the major drawback of poor bioavailability. The aim of this work was to evaluate the potential of AmB-loaded cubosomes as an oral formulation with improved bioavailability. This manuscript firstly developed AmB-loaded cubosomes by using the SolEmuls technology. The encapsulation efficiency, the *in vitro* release, and stability studies in simulated gastrointestinal fluid were used to evaluate AmB-loaded cubosomes. The acute nephrotoxicity, bioavailability, and tissue distribution study of AmB-loaded cubosomes were assayed upon oral administration to rats. SAXS and cryo-TEM exhibited AmB-loaded cubosomes as a bicontinuous cubic liquid crystalline phase with Pn3m geometry. The encapsulation efficiency and the results of *in vitro* release and stability studies in simulated gastrointestinal fluid further demonstrated that AmB was successfully encapsulated in cubosomes. AmB-loaded cubosomal formulation orally administrated in rats did not show nephrotoxicity and its relative bioavailability was approximately 285% as compared to Fungizone®. The AmB-loaded cubosomal formulation presented an effective potential approach for enhancing the oral bioavailability of AmB.

KEYWORDS: amphotericin B; cubosomes; oral bioavailability; SolEmuls technology.

INTRODUCTION

Amphotericin B was introduced as an antifungal drug to combat systemic fungal infection in 1959. Since then, amphotericin B has been used in the clinic and is still regarded as a golden standard and a life-saving drug in the treatment of many severe fungal infections (1–3). However, the medical application of AmB is limited by its toxicity and poor solubility, necessitating hospitalization and parenteral administration (1). An effective and safe oral formulation of AmB will surely have significant applications in the clinic because it will reduce renal toxicity, shorten or avoid hospitalization, and increase patient satisfaction. Unfortunately, AmB exhibits unfavorable biopharmaceutical properties of low solubility and permeability, resulting in poor absorption when administered orally (4).

Advancement in the understanding of gastrointestinal tract and in particular how lipids are digested and processed within gastrointestinal lumen has led to some effective approaches to improve drug solubility and gastrointestinal permeability. Several oral lipid-based formulations have been undertaken to facilitate AmB uptake across gastrointestinal tract in the past decades. As most of the attempts failed, the oral cochleate formulation of AmB developed by Biodelivery Sciences, Inc (USA) stands out to effectively facilitate the oral absorption and provide protection of AmB from gastric acid degradation or prevent P-gp efflux. More encouragingly, a favorable outcome of AmB phase I trial was released in February 2009 and the company emphasized that further plans of AmB clinical trials would be underway (5). Another oral lipid-based AmB formulation is currently in development by iCo Therapeutics Inc (Canada), which uses proprietary mixture comprised of AmB, DSPE-PEG, monoglycerides, and diglycerides (Peceol®), resulting in a 50-fold increase in AmB solubility over its conventional lipid formulation. The lipid nature of the formulation protects the acid labile AmB from destruction in the stomach and thus results in a positive therapeutic effect equivalent to intravenous therapy (4,6,7). In addition, these two oral lipid-based formulations, AmB cochleate and AmB in Peceol/DSPE-PEG, were successful in reducing the nephrotoxicity compared to AmB intravenous infusion. Concerning the above two lipid-based formulations developed by iCo Therapeutics Inc and BDSI company, no clinical application in the last 2 years was further reported and it is still very difficult to predict whether the lipid formulation will be developed into a successful oral product.

Cubosomes as novel lipid-based delivery vehicles have currently been highly recommended for active molecules due to their low viscosity, large interfacial areas, and the presence of both hydrophilic and hydrophobic regions (8). Cubosomes are nanoparticles, more accurately nanostructured liquid-crystalline particles (8), in a liquid-crystalline phase with cubic

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crystallographic symmetry formed by self-assembly of amphiphilic or surfactant-like molecules. Cubosomes could effectively improve drug bioavailability upon oral administration. For example, oral administration of cubosomes loaded with insulin resulted in a sustained reduce of blood glucose levels in rats (9).

Nevertheless, in the face of significant physicochemical barriers and a collection of failed attempts, the pervasive attitude toward the impossibility of developing a practicable oral AmB formulation emerged. As the patient population requiring AmB therapy is increasing, science need rise to meet the challenge. In the previous study, optimizing the preparation method for AmB-loaded cubosomes had been reported by our group (10). Therefore, the research was further to evaluate the *in vitro* characterization of AmB-loaded cubosomes, such as particle size, *in vitro* release and stability in simulated gastric and intestinal fluid. The research investigated the oral bioavailability of AmB in SD rats as a potential oral formulation. In addition, SolEmuls technology theory was firstly used to illustrate the development of AmB-loaded cubosomes (10).

MATERIALS AND METHODS

Materials

Amphotericin B was obtained from North China Pharmaceutical Group Corporation (Shenyang, China), and Fungizone® was a gift sample from the same company. Phytantriol was purchased from DSM (Basel, Switzerland). Poloxamer 407 ($PEO_{98}POP_{67}PEO_{98}$) was a gift from BASF (Ludwigshafen, Germany). Deoxycholate sodium was purchased from Jingxin Biological Technology (Guangzhou, China). Dialysis bag with a molecular weight cutoff (MWCO) of 3,500 Da was purchased from SMI (Vancouver, Canada). Pancreatic enzymes were obtained from Qiyun Biological Technology (Guangzhou, China). Acetonitrile (high-performance liquid chromatography (HPLC) grade) was purchased from SK Chemicals (Seoul, Korea). Milli-Q grade water purified through a Millipore system (ELGA LabWater, Sartorius, UK) was used throughout this study.

Preparation of AmB Deoxycholate

In 30 ml of deionized water, 0.50 g of AmB and 0.41 g of deoxycholate were dissolved. Subsequently, sodium hydroxide solution was gradually added until AmB completely dissolved into the solution. After the solution volume was diluted to 100 ml with 0.01 M, pH 6.2 phosphate buffer solution, yellow powder of AmB deoxycholate was obtained by a freeze-drying method.

Preparation of AmB-Loaded Cubosomes

AmB-loaded cubosomes were prepared through fragmentation of the Phytantriol/poloxamer 407 (PYT/P407) bulk cubic gel. PYT and P407 at a ratio of 18:1 (*w*/*w*) were melted at 60°C in a hot water bath till homogenous. Then, AmB deoxycholate (equivalent to 60 mg of AmB) was dissolved in 2 ml of deionized water. Subsequently, the AmB deoxycholate solution was added gradually into the PYT/P407 melt, followed by 1 min of vortex mixing to achieve a homogenous state. The samples were equilibrated at room temperature for 48 h, and then optically isotropic cubic-phase gels were formed. Subsequent fragmentation in 20 ml of water was performed by intermittent probe sonication (JYD-650, Shanghai, China) for 10 min using a pulse mode (9 s pulses interrupted with 18 s breaks) at 400 W energy input. The resulting milky coarse dispersions were homogenized using a high-pressure homogenizer (Avestin Em-C3, Ottawa, Canada) at certain high pressures and cycles to obtain an opalescent dispersion of the cubosomes. The final dispersions of cubic-phase gel were stored at room temperature for later studies.

Particle Size

The size distribution of cubosomes was measured using a Zetasizer 2000 PCS (Malvern Instr., Malvern, UK) which operated on the principles of laser diffraction (0.02–2,000 μm). The cubosome samples were diluted with purified water to 10% of light shading percentage before measurement. Cubosomal size was calculated by using an irregular sphere model and analyzed by the Dispersion Technology Software provided by Malvern Instruments.

Encapsulation Efficiency

With the aim to quantify AmB content encapsulated in cubosomes after production, 0.5 ml of cubosomes containing AmB were added into the reservoir of centricon (YM-100, Amicon, Millipore, Bedfore, MA, USA) (9). After centrifuging the cubosome dispersion at 15,000 rpm for 30 min, the filtrate which contained free AmB was removed. Then, the filtered dispersion was diluted with methanol and analyzed for entrapped AmB content using HPLC. A mixture of 0.01 M, pH 6.2 phosphate buffer solution and acetonitrile (60:40, *v*/*v*) was used as the mobile phase at a flow rate of 1.0 ml/min. AmB was separated by Phenomenex Luna C18 column (5 μm, 4.6×250 mm, Torrance, CA, USA) at 35°C and detected at 406 nm.

Cryogenic Transmission Electron Microscopy

For cryogenic transmission electron microscopy (cryo-TEM) examination, 4 μl of cubosome sample was applied on a carbon-coated holey film grid, and then gently blotted with filter paper for about 3 s to obtain a thin liquid film on the grid. Immediately after blotting, the grid was plunged into pre-cooled liquid ethane for flash freeze. Then the cryo-grid was held in a Gatan 626 Cryo-Holder (Gatan, USA) and the sample was transferred into a cryo-transmission electron microscope (JOEL JEM-2010, Tokyo, Japan) at −172°C. Samples were observed under minimal dose condition and the images were recorded digitally by a CCD camera (Gatan 832) at the defocus of 3–5.464 μm.

Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) measurements were carried out on a high-flux SAXS instrument (SAXSess, Anton Paar, Graz, Austria) operating in line collimation and equipped with an imaging plate (IP) as a detector. The IP with

a pixel size of 42.3×42.3 μm^2 was extended into a wide-angle range (the q range covered by the IP was up to 28 nm^{-1}, $q=(4\pi\sin\theta)/\lambda$, where λ is the wavelength of 0.1542 nm and 2θ is the scattering angle). The liquid samples were carefully loaded into a quartz capillary with a diameter of 1 mm. The exposure time was 60 min for the liquid samples.

In Vitro Release

The *in vitro* release behaviors of AmB-loaded cubosomes and AmB deoxycholate at the same concentration were evaluated using a membrane dialysis method. Two milliliters of samples were dispersed in 1 ml of release medium, which consisted of 0.25% *w*/*v* of sodium lauryl sulfate, in dialysis bags (MWCO 3,500 Da) and sealed. The dialysis bags were then immersed, using an orbital shaking water bath, into 10 ml of release medium maintained at 37°C and a revolution speed of 100 rpm. At specified time intervals of 1, 2, 4, 8, 12, and 24 h on the first day followed by 24-h interval for the subsequent days, the release medium was completely collected and replaced with fresh release medium. AmB content in the collected release medium was assayed using the HPLC method similar to that used in encapsulation efficiency determination.

Stability in Simulated Gastric and Intestinal Fluid

Into 50 ml of simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) in the screw-capped tubes, 0.5 ml of AmB-loaded cubosomes was placed. SGF without enzymes was composed of 30 mM NaCl, titrated to pH 1.2 with 1 N HCl. SIF was composed of 0.2 MNaOH, 6.8 g/L monobasic potassium phosphate and 10 g/L pancreatin, adjusted to pH 7.5 with NaOH. The sample tubes were incubated at 37°C in an orbital shaking water bath and stirred at 100 rpm in the dark. At predetermined time intervals of 30, 60, 120, and 180 min, 0.5 ml of SGF or SIF medium was drawn, and after each sampling, 0.5 ml of fresh release medium was added in the tubes for compensation. After completely solubilizing the 0.5 ml sample in methanol for clarification, the amount of AmB was determined by HPLC method as described above.

Animal Studies

In vivo studies of AmB-loaded cubosomes were carried out in male Sprague Dawley rats weighing between 180 and 200 g (Guangzhou University of Chinese Medicine, Guangzhou, China). The rats were housed in an animal care facility under standard condition of controlled temperature and humidity, a 12-h light–dark cycle. The animals were kept on standard pellet diet and water was made available *ad libitum*. The rats were cared for and handled according to the national regulations for experimental animals and the Ethical Committee of Sun Yat-sen University.

Eighteen rats were randomly divided into three treatment groups with six rats per group. Group I was orally treated with Fungizone® equivalent to 10 mg/kg AMB. Group II was orally administered with AmB-loaded cubosomes equivalent to 10 mg/kg AMB and group III was orally treated with AmB-loaded cubosomes at the dose of 20 mg/kg body weight.

Nephrotoxicity Study

The rats were fasted for 6 h first for oral treatments; 0.5 ml of blood samples were drawn from the retro-orbital plexus prior to and 24 h after the oral gavage. Plasma was immediately harvested by centrifugation and analyzed for blood urea nitrogen (BUN) and plasma creatinine (PC) using commercially available kits (Leadman Biochemistry Co., Ltd, Beijing, China) (10,11).

Pharmacokinetics Analysis

After administration of a single oral dose, blood samples were collected from the retro-orbital plexus into the heparinized micro-centrifuge tubes under mild ether anesthesia at 1, 2, 4, 8, 12, and 24 h on the first day and then every 24 h for four more days. Plasma was obtained by centrifugation at 8,000 rpm for 10 min. Then, 200 μl of methanol/acetonitrile (1:1 *v*/*v*) mixture was added into 100 μl of plasma for precipitating the plasma proteins. The samples were vortexed for 1 min and centrifuged at 16,000 rpm for 10 min. Finally, 25 μl of the resulting supernatants were injected into the HPLC for determining AmB content.

Tissue Distribution

On the fifth day following administration of AmB, each rat was humanely sacrificed and the liver, kidney, spleen, heart, and lung were removed, dried, and weighed; 0.5 g of each tissue sample was homogenized into 1 ml of normal saline in an ice bath. Two parts of methanol/acetonitrile (1:1 *v*/*v*) were added to one part of the homogenate for precipitating the plasma proteins. The resulting mixtures obtained from liver, kidney, spleen, heart, and lung were vortexed for 1 min and centrifuged at 16,000 rpm for 10 min. Finally, 25 μl of the resulting supernatants were injected into the HPLC for determining AmB content.

HPLC Analytical Assay

HPLC method was slightly modified for determination of AmB concentration (11,12). Briefly, 25 μl of the extracted sample was injected into a Phenomenex Luna C-18 column at 35°C. AmB was eluted at a flow rate of 0.5 ml/min with the mobile phase consisting of 0.01 M, pH 6.2 phosphate buffer solution and acetonitrile (60:40, *v*/*v*). The effluent was monitored at 406 nm and retention time was 10 min. AmB concentration in the sample was calculated using an external calibration curve of AmB in methanol. A limit of qualification of 10 ng/ml was obtained under the conditions described above.

Statistics

Pharmacokinetic parameters were determined by using 3p97 computer software (Chinese Association of Mathematical Pharmacology, Beijing, China). Statistical significance in the difference of the means was evaluated by using the Student's *t* test.

RESULTS

Particle Size Distribution of AmB-Loaded Cubosomes

As previously reported for homogenization to form cubosomes, a few particle aggregates in the micrometer size range could be found microscopically (13,14). The loss of cubosomal formulation would be taken into account when the sample was filtered through mixed esters cellulose membrane to remove big cubosomal aggregates. To produce cubosomes with lowering the presence of larger irregular particles, the influence of homogenization conditions on the cubosomal size distribution was investigated in this study.

The particle size of cubosomes prepared was characterized by Zetasizer 2000 PCS (Table I). During the sonication procedure, the cubic phase gel was efficiently disrupted and the mean particle sizes of the dispersions were spread in a wide range from 315 to 2,540 nm. Following homogenization at different pressures and with different cycles, the mean diameter of cubosomes was dispersed from a broad size distribution to a narrow lognormal size distribution, indicating the high shear energy homogenization was a powerful technique to convert large particles into smaller ones. Starting from 600 bar homogenization pressure and three homogenization cycles, the mean *z*-average diameter of the coarse cubosomes was 1,875 nm with quiet a few large particles. When changing the homogenization conditions to 1,200 bar and nine cycles, the size of the homogenized samples was efficiently reduced to 315 nm with more uniform size distributions as a result of increased energy input. Table I illustrated that cubosomes with narrow particle size distribution were obtained under homogenization condition of 1,200 bar and nine cycles, while lower homogenization pressures and fewer cycles produced cubic nanoparticles with broad diameter distribution.

Encapsulation Efficiency and Drug-Loading Rate

The encapsulation efficiency of AmB-loaded cubosomes was determined over 87.8%, indicating most of AmB was encapsulated in cubosomes.

The maximum dose of AmB deoxycholate dissolved in 2 ml of water was equivalent to 60 mg AmB. As a result, 60 mg AmB were loaded into the cubosomal formulation consisting of 1.8 g of PYT and 0.1 g P407. Then, drug loading ratio was determined by centrifuging the drug-loaded cubosomes. AmB-loaded cubosomes with the maximum drug-loading rate of 3.0% could be obtained for the following studies.

Cryo-TEM Characterization

Cryo-TEM was carried out to investigate the internal structure of AmB-loaded cubosomes. The cryo-TEM images (Fig. 1) showed the cubosomes were generally in spherical shape, around 200–300 nm in size, with a rough nodular surface as described previously for cubosomes prepared by fragmentation (15). The insert in Fig. 1 showed a typical ordered cubic texture with a spacing of repeat unit for AmB-loaded cubosomes. The spot in Fig. 1 indicated the diameter of the intercalating cubosomal aqueous channel, showing the cubic phase nanoparticles still maintained a well-defined inner morphology and a fraction of small vesicular particles was effectively converted to cubic phase particles. It was observed in Fig. 1 that particle size of the cubosomes ranged with a mean size of about 300 nm, which was consistent with the particle size results measured by Zetasizer (Table I).

X-ray Diffraction Analysis

Small-angle X-ray scattering examination was performed on AmB-loaded cubosomes with the aim to determine the structural organization. Three distinct Bragg peaks were observed on the X-ray diffraction profiles with the relative positions at ratios of $2^{1/2}$:$3^{1/2}$:$4^{1/2}$, relatively (10). This was in accordance with the bicontinuous cubic-phase structure with Pn3m space group, indicating the cubosomes had a D-type cubic nanostructure. No peaks for the small vesicles were observed. In agreement with cryo-TEM images, X-ray diffraction measurements showed that the sample consisted of a large fraction of particles with inner cubic structure and a negligible fraction of small vesicles.

In Vitro Release

Considering the poor aqueous solubility of AmB, 0.25% *w*/*v* sodium dodecyl sulfate (SDS) solution was used as a dissolution medium and a sink condition was maintained (16). In Fig. 2, the release of AmB from AmB-loaded cubosomes was compared with that from AmB deoxycholate. After 96 h, approximately 61% of AmB was released from AmB deoxycholate, while only around 12.3% of the entrapped AmB was released from AmB-loaded cubosomes. The release rate of AmB-loaded cubosomes was obviously lower than that of AmB deoxycholate ($p<0.05$). The poor dissolution rate of AmB-loaded cubosomes over the study period of 96 h could attribute to the fact that the incorporation of AmB in cubosomes made it difficult to be released in this kind of medium.

Table I. Mean Particle Size of Three Batches of Cubosomes Measured by Zetasizer 2000 PCS (Data Represented as Mean ± SD, $n=3$)

Cubosomes (high-energy dispersion) (μm)	Surface area mean particle size	d(0.1) (μm)	d(0.5) (μm)	d(0.9) (μm)
Ultrasound 600 bar	2.540±0.100	1.195±0.053	3.465±0.140	13.169±1.645
Homogenization for 3 cycles 600 bar	1.875±0.0146	1.0257±0.052	2.327±0.020	4.474±0.508
Homogenization for 6 cycles 600 bar	1.708±0.018	1.034±0.007	1.962±0.039	3.366±0.148
Homogenization for 9 cycles 600 bar	1.488±0.021	0.938±0.009	1.674±0.032	2.708±0.054
Homogenization for 3 cycles 1,200 bar	0.642±0.076	0.244±0.036	1.167±0.058	3.052±0.142
Homogenization for 6 cycles 1,200 bar	0.394±0.016	0.181±0.007	0.543±0.045	2.032±0.328
Homogenization for 9 cycles 1,200 bar	0.315±0.051	0.170±0.012	0.344±0.115	1.297±0.107

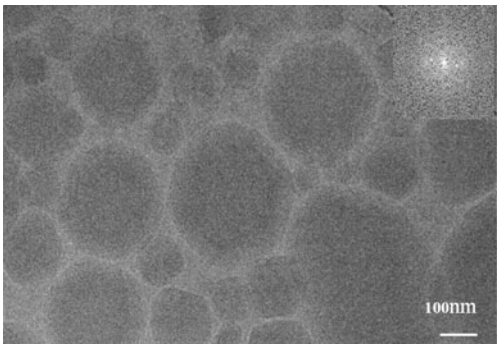

Fig. 1. Cryo-TEM micrographs of AmB-loaded cubosomes

This result also provided a direct evidence that AmB was incorporated into cubosomes.

Stability in Simulated Gastric and Intestinal Fluid

The chemical stability of AmB-loaded cubosomes was evaluated in SGF as well as SIF with pancreatin. The results in Fig. 3 showed that only 25.0 and 21.7% of AmB was measured for AmB deoxycholate and AmB-loaded cubosomes in SGF for 3 h respectively. Comparing AmB-loaded cubosomes with AmB deoxycholate, no significantly difference in drug degradation rate was observed ($p>0.05$). Quick sedimentation of AmB deoxycholate was observed in SGF, while AmB-loaded cubosomes remained as dispersed state in SGF. The aggregate state of AmB deoxycholate definitely retarded the dissolution and degradation of AmB, so AmB deoxycholate in SGF was not a proper control for comparison.

In evaluating the degradation of AmB in SIF, it was noted that both AmB-loaded cubosomes and AmB deoxycholate in SIF remained in good dispersion state in 3 h. After 3 h incubation in SIF, it was observed that AmB-loaded cubosomes showed 74.0% detectable AmB while only 49.2% AmB was detected in AmB deoxycholate (Fig. 4). This suggested a remarkably better stability of AmB-loaded cubosomes in SIF as compared to AmB deoxycholate. The improved stability could be due to the incorporation of AmB into cubosomes significantly reducing the exposure of AmB to SIF and decreasing the degradation.

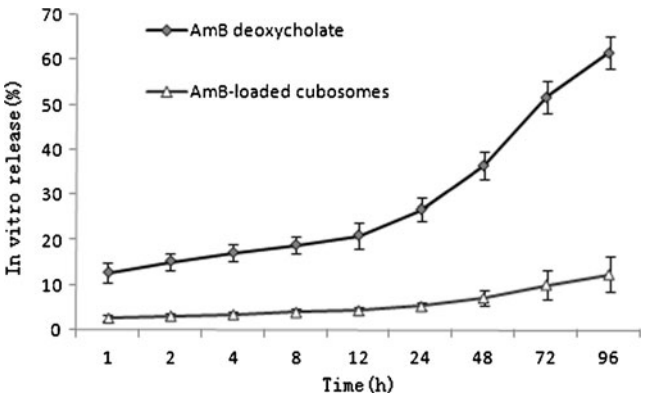

Fig. 2. *In vitro* release profiles of AmB-loaded cubosomes in SDS solution (data represented as mean ± SD, $n=6$)

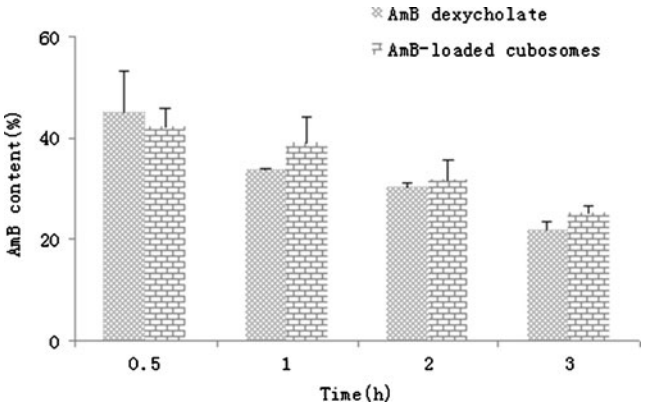

Fig. 3. Stability of AmB in SGF (data represented as mean ± SD, $n=6$)

ANIMAL STUDIES

Nephrotoxicity Study

The most severe adverse effect of AmB lies in its nephrotoxicity (16). As the two reliable markers of the clinical renal function, BUN and PC levels were determined to evaluate AmB acute nephrotoxicity upon oral administration of high dose (16). Abnormal rise in either BUN or PC levels indicates the dysfunction or damage to the kidney.

Plasma BUN and PC concentrations prior to and 24 h following oral administration of AmB-loaded cubosomes (10 or 20 mg/kg) were detected and no statistically significant changes were found before and after the administration (Table II). Additionally, the BUN and PC levels for those orally administrated with AmB-loaded cubosomes were comparable to those administrated with Fungizone® (10 mg/kg). The results in Table II were consistent with the previously reported data, indicating the *in vivo* nephrotoxic potential was not observed following oral administration of AmB-loaded cubosomes (16,17).

Pharmacokinetics Analysis

AmB-loaded cubosomes were designed to improve the oral bioavailability of AmB. Blood levels after oral administration of AmB-loaded cubosomal formulation at doses of 10

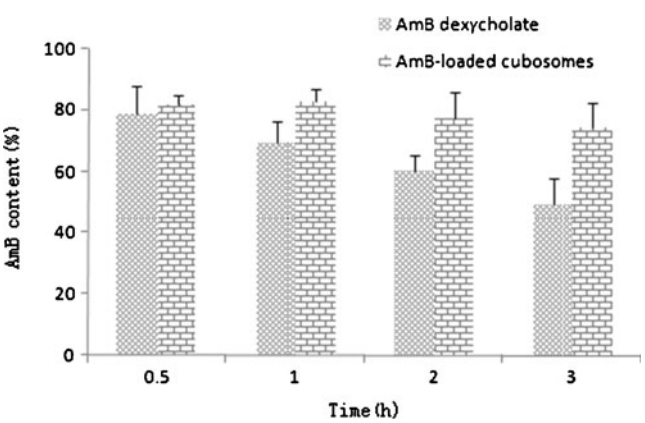

Fig. 4. Stability of AmB in SIF (data represented as mean ± SD, $n=6$)

Table II. Plasma BUN and PC Concentrations Following Oral Administration of AmB (Data Represented as Mean ± SD, n=6)

	BUN (mg/dl)		PC (mg/dl)	
Formulation	0 h	24 h	0 h	24 h
Fungizone® (10 mg/kg)	5.7±1.2	5.3±0.6	47.9±7.2	48.9±6.5
AmB-loaded cubosomes (10 mg/kg)	7.8±1.6	7.0±1.9	45.9±4.1	46.2±7.3
AmB-loaded cubosomes (20 mg/kg)	6.4±0.9	6.3±0.9	50.5±2.8	48.2±4.1

and 20 mg/kg were compared with those after oral administration of AmB deoxycholate at doses of 10 mg/kg. The major pharmacokinetic parameters obtained were listed in Table III, including C_{max}, T_{max}, and AUC. The mean plasma levels of AmB after a single oral administration with three formulations were shown in Fig. 5.

In Table III, the AUC value for oral administration of AmB-loaded cubosomes at 10 mg/kg dose was significantly higher than that administrated with oral Fungizone®, indicating the relative bioavailability of the cubosomal formulation was increased to 285%. As seen in Fig. 5, plasma drug profile of AmB-loaded formulations showed a sustained release of AmB over a period of 5 days, while the plasma concentration of Fungizone® could be detected for only 2 days. The high dose of AmB-loaded cubosomes (20 mg/kg) led to more drug release in the plasma. However, the low dose of AmB-loaded cubosomes (10 mg/kg) at 4 h following the oral administration was found to generate a higher initial plasma concentration of AmB up to 165.5±58.2 ng/ml *vs.* 131.7±13.0 ng/ml for high dose of cubosome formulation. At 8 h after the administration, the plasma concentrations of AmB reached equivalence for the low and high doses of cubosomes and after 12 h the plasma concentrations of high dose exceeded those of low dose. At 120 h following the administration, the concentration of AmB could still be detected in the case of the high-dose cubosomes. It should be noted that since rats do not have gall bladder, they are unable to digest rapidly and efficiently a large volume of lipids. Concerning the slower absorption rate as a result of the larger volume of administered lipids, the results may attribute to delay of gastric empting and slow peristaltic movements in the rat model.

Tissue Distribution

The results of tissue distribution of AmB were summarized in Fig. 6 following oral gavage administration of AmB-loaded cubosomes at doses of 10 and 20 mg/kg and Fungizone® at 10 mg/kg. Fungizone® showed relatively homogeneous distribution among liver, kidney, spleen, heart, and lung. Similarly, orally administered AmB-loaded cubosomes showed relatively homogeneous AmB distribution among tissues, with a significant shift towards the kidney in the case of 20 mg/kg dose, but not in the case of the lower dose (10 mg/kg).

DISCUSSION

Some efforts were made in the past years to improve the oral bioavailability of AmB (4). AmB solution, nanosuspension, PLGA nanoparticles, and lipid nanospheres (5,6,16). However, most of the studies were still in a potential possibility for developing the oral formulation. As most of the attempts failed, the pervasive attitude toward the impossibility of developing a practicable oral AmB formulation emerged. More encouragingly, the oral AmB cochleate formulation was under development by Biodelivery Sciences, Inc. (BDSI), releasing the favorable outcome of phase I trial in February 2009 (5). Another oral lipid-based AmB formulation was also in development by iCo Therapeutics Inc (Canada), resulting in a 50-fold increase in AmB solubility over its conventional lipid formulation (5). Compared with the other drug-delivery systems, the nature of the lipid formulation is similar to the structure of cell membrane. Thus, oral lipid-based formulations could best facilitate AmB uptake across gastrointestinal tract and best protect the acid labile AmB from destruction in the stomach. As a result, these two oral AmB lipid-based formulations led to a positive therapeutic effect equivalent to intravenous therapy as reported (5). Cubosomal formulations are considered as another lipid-based delivery vehicle that has the potential possibility for delivery of AmB, because of their multilayer structures consisting of continuous lipid bilayers which are similar to those of cochleates. In addition, it is likely the cubic phase structure without a molecular ester bond can be retained under the digestive conditions while cochleates consisted of lipid composition have the digestion process in the gastrointestinal tract. In this study, AmB-loaded cubosomes as an oral formulation were developed to enhance the solubility and gastrointestinal permeability of AmB. However, due to the low solubility of AmB in PYT (less than 1 mg/g), it is difficult to develop AmB-loaded cubosomes as a therapeutically practicable oral drug delivery system.

Table III. Pharmacokinetic Parameters Upon Oral Administration of Fungizone® and AmB-Loaded Cubosomes (Data Represented as Mean ± SD, n=6)

Formulation	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-120} (hng/ml)
Fungizone® (10 mg/kg)	218.5±30.9	6.3±2.3	21.4±6.1	5,964.0±705.4
AmB-loaded cubosomes(10 mg/kg)	316.7±32.6	26.0±4.9	20.0±4.2	17,002.2±3,400.3
AmB-loaded cubosomes (20 mg/kg)	461.6±67.6	26.0±4.9	26.9±9.0	20,946.3±3,310.7

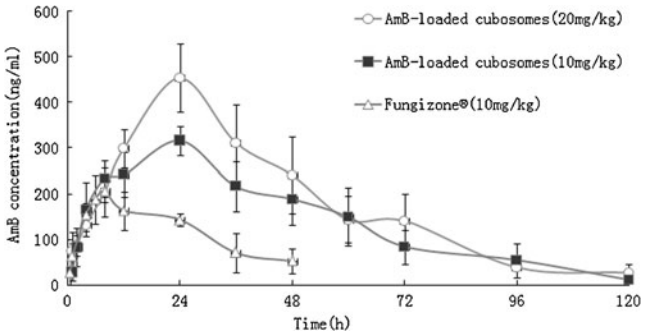

Fig. 5. Comparative *in vivo* plasma concentration *vs.* time profiles following a single dose of oral administration (data represented as mean ± SD, n=6)

SolEmuls technology is used for incorporating the poor-water-soluble drug in emusions by now. However, it is unknown whether this technology could be used to the cubosomal formulation. This manuscript was firstly reported that the poorly soluble drug in both water and oil could successfully be incorporated in cubosomal formulation by this technology. The research of SolEmuls technology in AmB emulsion preparation may lead to better understanding how to improve the AmB-loaded cubosomes (18).

Theoretically, AmB could spontaneously diffuse from the aqueous phase into the emulsion phase according to its partitioning coefficient. However, the rate-limiting step is the dissolution of AmB from the crystals, as it is commonly known that the poor solubility of AmB may simultaneously lead to a slow dissolution. So, localizing AmB in the emulsion phase appeared feasible to achieve if the dissolution rate from AmB crystals was accelerated. To enhance the dissolution rate it is firstly required to provide surface areas as large as possible which means using drug crystals as small as possible. Secondly, during the homogenization, high speed streaming, i.e. a kind of 'supersonic' stirring, is generated which could lead to a fast dissolution of drug crystals and rapid partition of drug molecules into lecithin layer (19–21). This AmB emulsion preparation process applying SolEmuls technology was described as below (18). Briefly, either a jet-milled drug powder or, preferentially, a drug nanosuspension was mixed with a coarse pre-emulsion such as Lipofundin. Upon homogenizing the pre-emulsion, AmB emulsion was achieved.

Based on the SolEmuls theory and preparation process, the AmB cubosomes preparation could also attribute to a large surface area by AmB deoxycholate and fast diffusion gradient by homogenization. Compared to AmB powder, the particle size of AmB deoxycholate as colloidal dispersion changed from micrometer to nanometer, which resulted in efficient incorporation of AmB into cubosomes under homogenization. It is important to incorporate the maximum drug concentration to minimize the lipid amount for oral formulation. Based on the oral doses of 15 mg/kg AmB provided by BDSI company, the AmB-loaded cubosomes with 3% of drug loading efficiency could meet the need for developing oral formulation as the corresponding lipid dose of 25 g PYT/50 kg is generally acceptable.

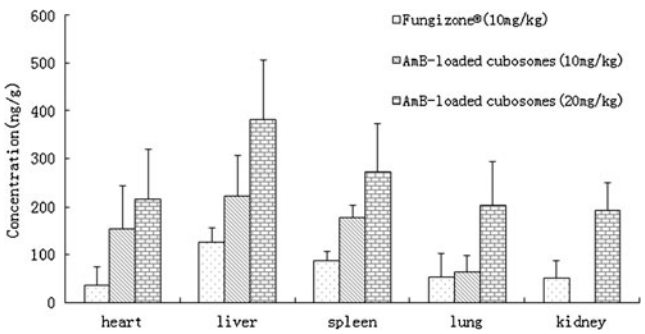

Fig. 6. Tissue distribution of AmB at 24 h following oral administration (data represented as mean ± SD, n=6)

In fact, by adding amphotericin solution Fungizone® into the parenteral emulsions, a classical precipitation of amphotericin crystals occurred as a solvent (Fungizone®) added into a non-solvent (water phase of emulsion). The precipitated crystals could fully dissolve only after intensive shaking for 18 h at a frequency of 2,800 rpm (22). However, in the preparation of AmB-loaded cubosomes, after homogenization no any visible crystals were observed under cryo-TEM, indicating that complete dissolution of AmB in PYT was achieved and drug molecules were incorporated into cubosomes. The cubosomal diameters, D50% and D99%, were 0.34 and 1.29 μm, respectively, measured on the day of production and showed no change after 1 month of storage at room temperature. The results further confirmed the incorporation of AmB into cubosomes prevented the drug precipitation in non-solvent.

In the previous report about cubosomal preparation, drug usually was added into the melted PYT or Glyceryl monooleate (GMO) and stirred continuously till the complete dissolution (14,23,24). To date, nothing has been done to improve the poor solubility of drug in PYT or GMO for developing cubosomal formulations. It was unknown whether micellar system (such as AmB deoxycholate) could be used to improve the drug solubility for cubosomal formulations. In the present research, the successful incorporation of AmB in cubosomes using AmB deoxycholate micelles presented an effective method to form cubosomes for the other poor-water-soluble or lipid-soluble drugs. This finding demonstrated that cubosome preparation followed the rule of solution preparation "like dissolves like" and this is very helpful for choosing the encapsulated drugs for cubosomal formulation. Also, the high pressure homogenization was proved to accelerate drug redistribution.

A significant increase (p<0.01) in the AUC values of all the cubosomal formulations in comparison to that of oral Fungizone® distinctly indicated that cubosomal delivery systems could improve the oral bioavailability of AmB. It is well known that the passive diffusion process is dependant on the drug molecular weight and the absorption efficiency decreases when the drug molecular weight over 500 Da. Upon oral administration of free form (as presented in Fungizone®), AmB is mainly absorbed by passive diffusion through intestinal membrane (5). However, with molecular weight of 924.08 Da, the passive diffusion of AmB should be very low. In addition, AmB is subject to chemical degradation at acidic environment in gastrointestinal tract and could be degraded in GIT prior to its absorption upon oral administration of Fungizone® (17). These manuscripts reported that the advantages of cubosomes as oral delivery system may lie in the following aspects (14,23,24). Firstly, the unique liquid-crystalline structure of cubosomes could provide protection for entrapped

drug from degradation in the gastrointestinal tract (25). Secondly, the lyotropic property of cubosomes with hydrophilic surface makes the contact with endothelial cell membrane more easily and overcomes the "unstirred water layer" barrier (26–28). Thirdly, the penetration of either cubosomes or the encapsulated drug molecules across the endothelial cell membrane is enhanced, which will increase the uptake efficiency of orally administrated drug (29). Therefore, to some extent, incorporation of AmB into cubosomes could provide protection for the drug from degradation in GIT and enhance the solubility of AmB in the intestinal lumen. Another explanation for improving the permeability of AmB-loaded cubosomes may be ascribed to promoting the lymphatic drug transport and affecting the fluidity of the enterocyte membrane (7). These absorption mechanisms could be accountable for the improved oral bioavailability of AmB (16).

AmB could be solubilized in the hydrophobic domains of cubic nanoparticles and might remain in the solubilized state even in the gastrointestinal tract, so that aggregation of AmB in SGF was not observed. This addressed that cubosomes could enhance the stability of AmB in gastrointestinal tract and be used as oral delivery system. In addition, the fate and absorption mechanism of AmB-loaded cubosomes *in vivo* need to be elucidated in future research.

In the study of tissues distribution, the relatively high uptake of AmB was found in spleen and liver, compared to heart, lung, and kidney. The possible reason is that liver and spleen are the end-points of the RES particle removal from blood. Compared to the low dose of cubosomal administration (10 mg/kg), the distribution of AmB-loaded cubosomes administered orally at a high dose of 20 mg/kg showed a significant shift towards the kidneys. The possible explanation was that dose of AmB-loaded cubosomes may play a key role in the kidney distribution. The correlation between AmB-loaded cubosomes dose and kidney distribution need to be elucidated in future research. This result also made it suspectible that orally administered AmB-loaded cubosomes might not able to reduce the nephrotoxicity of AmB. Similar result was also reported in a previous literature (7). It was pointed out that oral administration of AmB emulsion could still provide a chance to eliminate its nephrotoxicity. Once AmB reached the tissues, it remained there for a prolonged time, slowly 'leaking' back to the bloodstream and subsequently being slowly eliminated from the body. This hypothesis of prolonged storage of AmB in the tissues was also supported by previously published works and multicompartmental analysis of the data (30,31). Therefore, oral administration was completely different from i.v. administration, in which high initial concentrations in the target tissues are reached, leading to greater chances of related toxicity. Apart from the hypothesis, in this study no significant changes in plasma BUN and PC concentrations were observed prior to and 24 h following oral administration of AmB-loaded cubosomes even at a high dose of 20 mg/kg, which demonstrated the decrease of AmB nephrotoxicity like the previously reported data (16).

CONCLUSIONS

It was firstly demonstrated that the encapsulation of drug in cubosomes followed the rule of "like dissolves like" and homogenization could accelerate drug redistribution during the process of cubosomal preparation. AmB-loaded cubosomes exhibited very exciting progress towards the development of potential formulation for oral administration as the cubosomal formulation demonstrated effectiveness in improving the oral bioavailability of AmB and decreasing the drug nephrotoxicity.

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