Biodistribution of Amphotericin B When Delivered Through Cholesterol Hemisuccinate Vesicles in Normal and *A. fumigatus* Infected Mice

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Purpose. This study compared the biodistribution of two amphotericin B formulations in normal and *Aspergillus* infected mice. Amphotericin B cholesterol hemisuccinate vesicles (ABCV) which reduces the toxicity of amphotericin B and thereby enhances its therapeutic efficacy in a murine model of aspergillosis was compared with conventional amphotericin B deoxycholate suspension (AmB_{DOC}).

Methods. ABCV (12 mg/kg wt) and AmB_{DOC} (2 mg/kg wt) were intravenously administered to normal and *A.fumigatus* infected mice. The concentration of amphotericin B in plasma and other organs was determined at different time points.

Results. It was observed that ABCV had a significantly different pharmacokinetic profile compared to conventional amphotericin B. In comparison to AmB_{DOC} significantly lower levels of amphotericin B were observed in kidneys and plasma, the major target organs of toxicity. Animals receiving ABCV demonstrated high levels of amphotericin B in liver (38% retention till 48 h) and spleen (2.6% retention till 48 h) in comparison to AmB_{DOC} (7.3% and 0.21% retention in liver and spleen respectively till 48 h). Biodistribution studies of ABCV in infected mice demonstrated that there was a moderate enhancement in levels of amphotericin B in liver, spleen, lungs and kidneys as compared to normal mice and the plasma levels were reduced. However, such observations were not made after AmB_{DOC} administration to infected mice except for kidneys in which there was a marked increase in uptake as compared to normal mice.

Conclusions. Our results suggest that prolonged retention of high concentrations of ABCV in reticuloendothelial system organs is the reason for its reduced toxicity. Enhanced localization of the drug at the infected site may lead to improvement in therapeutic efficacy.

KEY WORDS: aspergillosis; pharmacokinetics; amphotericin B; biodistribution; liposomes; cholesterol hemisuccinate.

INTRODUCTION

Amphotericin B, a polyene antibiotic, has been widely used to treat life threatening fungal infections (1,2). But infusion related side effects and renal insufficiency associated with its administration have severely limited its therapeutic applicability (3,4). To circumvent its toxicity many lipid formulations of amphotericin B have been developed (5–8). It has been demonstrated that these lipid based formulations of amphotericin B like Amphotericin B lipid complex (ABLC) (9), Amphotericin B colloidal dispersion (ABCD) (10,11), and liposomal amphotericin B (AmBisome) (12), have reduced toxicity without significant loss of antifungal activity. All these formulations have altered pharmacokinetics and tissue distribution compared to the conventional deoxycholate formulation of amphotericin B (AmB_{DOC}) (10,13,14).

We have recently reported a novel lipid-based dosage form of amphotericin B called amphotericin B cholesterol hemisuccinate vesicle (ABCV) (15). It is prepared from amphotericin B and cholesterol hemisuccinate in 1:2 molar ratio. This formulation has significantly reduced nephrotoxicity and *in vitro* toxicity to erythrocytes compared to AmB_{DOC}. On the basis of maximum tolerated dose (MTD), it was found to be almost 9 times less toxic *in vivo* compared to AmB_{DOC}, but yet retained its antifungal activity against aspergillosis in Balb/c mice.

To determine whether the observed reduction in toxicity and improvement of therapeutic efficacy is due to altered pharmacokinetics and tissue distribution of amphotericin B when delivered through cholesterol hemisuccinate vesicles, we compared the biodistribution of ABCV to that of AmB_{DOC} in infected as well as normal mice.

METHODS

Chemicals

Cholesterol hemisuccinate, amphotericin B and AmB_{DOC}, the deoxycholate solubilised preparation of amphotericin B were obtained from Sigma Chemical Co. (St.Louis, MO, USA) in different purity levels. The equivalence of these two forms of amphotericin B were evaluated both spectrophotometrically as well as by HPLC and subsequently used for experimental studies. AmB_{DOC} was reconstituted in 5% dextrose in water to a final concentration of 160 μg/mL before use. 0.25 mL of this dose was administered in mice for tissue distribution studies.

Animal Model for *Aspergillus Fumigates* Infection

Male Balb/c mice (body wt., 20±2 g) obtained from the Laboratory Animal Facility of National Institute of Nutrition, India were used. All animal experiments were carried out with prior permission and in accordance with the standards of NIH as described in "Principles of Laboratory Animal Care ". Groups of animals were injected intravenously via tail vein with 0.25 mL saline containing 3.6×10^6 spores of *A. fumigatus.* From our previous studies we had known that sufficient dissemination of fungus to various organs takes place by 24 h, so we dosed the animals at this time in order to observe the difference in biodistribution due to infected state. From our previous work, we also knew that 12 mg/kg wt ABCV treatment could control fungal invasion whereas AmB_{DOC} treatment could not (15). Therefore we choose 12 mg/kg wt ABCV and 2 mg/kg wt AmB_{DOC} as contrasting treatments to study the pharmacokinetic profile during infection and cure.

Preparation of Cholesterol Hemisuccinate Vesicles Containing Amphotericin B

Amphotericin B and cholesterol hemisuccinate were mixed in various mole ratios (1:1 to 1:4) in an attempt to

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ABBREVIATIONS: ABCV, Amphotericin B cholesterol hemisuccinate vesicles; AmB_{DOC}, Amphotericin B deoxycholate preparation; ABLC, Amphotericin B lipid complex; ABCD, Amphotericin B colloidal dispersion; AmBisome, Liposomal amphotericin B; HPLC, High-Pressure liquid chromatography; $Na^{125}I$, Sodium Iodide (radioactive); DL, Deciliter; iv, intravenous; RBC, red blood cells.

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reduce the toxicity of amphotericin B. At 1:1 molar ratio we observed higher toxicity (MTD was 15 mg/kg wt) than 1:2 molar ratio (MTD was 17 mg/kg wt). At higher cholesterol hemisuccinate ratios, toxicity did not appreciably decrease, so we used 1:2 amphotericin B - cholesterol hemisuccinate ratios for further studies. Amphotericin B cholesterol hemisuccinate vesicles were prepared aseptically as described in our previous report (15). In brief, 16.66 μM of amphotericin B and 33.33 μM of cholesterol hemisuccinate were mixed in 10 mL methanol. Lactose , a cryoprotectant, was added to a final concentration of 10% in ABCV. The dry film obtained on rotary evaporation in a sterile laminar flow cabinet was hydrated with isotonic aqueous medium (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4), sonicated and French pressed (SLM Instruments, Inc. Rochester, New York, USA). It was then extruded through a 1.2 μm filter and the final concentration of amphotericin B in the ABCV suspension was observed to be approximately 6 mg/mL. As demonstrated later in 'Results', amphotericin B cholesterol hemisuccinate vesicles were observed to be unstable in terms of retention of an aqueous phase marker whereas amphotericin B was stably associated with the ABCV vesicles.

Determination of Size of ABCV

Dynamic laser light scattering measurements for determining the size of ABCV were performed using a Brookhaven 9000 instrument (Brookhaven, NY, USA) with a BI 200 SM goniometer as described earlier (15,16). The size of ABCV was determined from the diffusion of the particles and the representative size distribution spectra are shown in Figure 1. The mean diameter of ABCV, obtained from the volume distribution curves provided by the particle analyzer was found to be 252 nm.

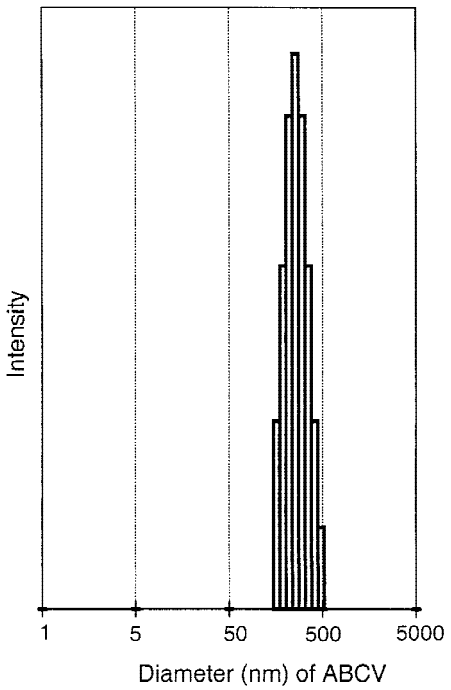

Fig. 1. Quasielastic laser light scattering data of ABCV. The mean diameter of ABCV is 252 nm.

High-Pressure Liquid Chromatography (HPLC) Analysis of Amphotericin B : Standard Curve and Recovery in Various Tissues and Plasma

The HPLC analysis of amphotericin B was done as described by Nilsson-Ehle *et al.* (17). The HPLC System consisted of a Gynkotek (Munich, FRG) Model 300°C high precision pump with a solvent delivery system, an injector with a 0.02 mL loop and a dual wave length UV detector (Gynkotek UVD - 160). A C_{18} reverse phase column (Product No 831915, Phase Separation Ltd., CT, USA) consisting of 5 μm octadecylsilyl particles (250 mm × 4.6 mm) was used for separation at room temperature and the absorbance was recorded at 345 nm. An isocratic solvent delivery system of 1 part of 0.0005 M EDTA and 4 parts of methanol was employed at a flow rate of 1.0 mL/min. A computer-integrator which provided visual representation of the detector response was used for the analysis of peak areas. For constructing standard curves, a solution of 1 mg of amphotericin B per mL of methanol was prepared from a common amphotericin B stock. Appropriately diluted amphotericin B (0.02 mL) was injected (in duplicate) into the C_{18} reverse phase column. Retention time was determined and the areas under the peaks were plotted against amount of drug to obtain a standard curve within the range of 5–50 μg/mL amphotericin B. We observed that within this range (5–50 μg/mL) there was less than 10% variation in the peak area obtained from repeated injections.

To determine recovery in tissues and plasma, groups of six undosed mice were sacrificed and blood and various organs were obtained. Pooled organs (0.5 g) and pooled plasma (0.5 mL) were homogenized in 2 mL methanol containing 12.5 μg amphotericin B for 1 min at RT in an overhead Potter-Elvehjem tissue grinder (Wheaton Instruments Ltd., NJ, USA). The homogenate was spun at 9000g for 15 min and supernatant was analyzed by HPLC. The peak areas obtained were compared with those of standard amphotericin B and it was observed that around 80% of amphotericin B present in the tissue homogenate could be detected.

Preparation of Aqueous Phase Marker– [^{125}I]Tyraminylinulin and Entrapment in ABCV

Preparation and radiolabelling of tyraminylinulin with $Na^{125}I$ was carried out according to methods described earlier (15). For entrapping [^{125}I]tyraminylinulin in ABCV, the dried film of amphotericin B-cholesterol hemisuccinate was hydrated with Tris-HCl buffer containing 1 mg/mL tyraminylinulin with trace amounts of [^{125}I]tyraminylinulin. The unentrapped tyraminylinulin was separated by subjecting the sample to three ultracentrifugations at 210,000g for 50 min each.

In Vitro Stability of ABCV in Plasma and Buffer

ABCV used for *in vitro* stability studies had a concentration of 6600 μg/mL amphotericin B and it was diluted 10 times before use. To obtain plasma, blood was drawn from male Balb/c mice in the presence of heparin (50 units/mL) and freshly used for *in vitro* studies. 1 mL aliquots of ABCV containing entrapped [^{125}I]tyraminylinulin were added to 6 mL of prewarmed (37°C) mouse plasma or Tris-HCl buffer. At different time intervals (0–48 h), 1 mL of incubation mixture was withdrawn and centrifuged at 210,000g for 50 min to

pellet down ABCV entrapped [^{125}I]tyraminylinulin. The radioactivity of the pellet as well as free [^{125}I]tyraminylinulin in the supernatant were measured to calculate the retention of [^{125}I]tyraminylinulin in ABCV. Similarly, to determine the percentage retention of amphotericin B in ABCV, amphotericin B was estimated in pellet and supernatant.

Tissue Distribution of Amphotericin B Delivered Through Deoxycholate Suspension and Cholesterol Hemisuccinate Vesicles in Normal and Infected Mice

For tissue distribution studies, normal and infected Balb/c mice were iv administered (via tail vein) with 2 mg/kg wt AmB_{DOC} or 12 mg/kg wt ABCV. For this, 0.25 mL of 960 μg/mL ABCV or 160 μg/mL AmB_{DOC} was administered 24 h after injection of spores. Six animals in each group were sacrificed using chloroform at 1 h, 8 h, 24 h and 48 h after administration of amphotericin B preparations. 0.5 mL blood was drawn by cardiac puncture and heparin (50 units/mL) was used to obtain plasma for estimation of amphotericin B by HPLC. Various organs (lungs, liver, spleen and kidneys) were removed and analyzed for amphotericin B on the same day without any freezing. For this, pooled organs and plasma were homogenized and centrifuged as described earlier and the supernatant was analyzed by HPLC. Its peak area was compared with amphotericin B standards to determine the concentration in the homogenate. From this concentration and the weight of the organ, the total amphotericin B content in each organ was determined. An internal standard of amphotericin B (6.25 μg/mL) was added while homogenizing the plasma and tissue samples so that the total amphotericin B (internal standard plus tissue content) was in the detectable range of the HPLC assay. This was especially required for organs with low uptake (e.g. lung). Its area was subtracted from the final peak area obtained. Since only 80% of amphotericin B present in the tissues could be detected, so on the basis of this we calculated the actual amount of amphotericin B that was present in the tissues.

Pharmacokinetic Calculations

Single-dose pharmacokinetic estimates were derived by non-compartmental techniques (18). A non-linear least-squares regression program (IGOR PRO Version 3; Wavemetrics, Inc., OR, USA) was applied to the postdistribution phase of the concentrations-time curves to generate an estimate of elimination rate constant ($K_{initial}$). Initial phase half life was calculated as $t_{1/2} = \ln(2)/ K_{initial}$. The area under the concentration-time curve from zero to infinity ($AUC_{0-\infty}$) was calculated by the trapezoidal rule using the IGOR PRO software. Peak concentrations (C_{max}) of amphotericin B and its corresponding time (T_{max}) in various tissues were defined on the basis of observed data points. Plasma clearance (CL) was calculated by the formula CL= dose/$AUC_{0-\infty}$. For determining volume of distribution which is the volume of central compartment (V_c), theoretical plasma concentrations at zero time (C_0) were obtained by extrapolation of the concentration-time curve ahead of one hour time point and it was calculated as V_c = dose/ C_0. Both plasma clearance and volume of distribution were expressed in per kg units.

RESULTS

In Vitro Stability of ABCV in Buffer and Plasma

The tendency of lipid vesicles to lose their integrity on interaction with plasma components is influenced by their composition (19). In order to evaluate the integrity of this sterol vesicle, the stability of ABCV in the putative physiological environment comprising the plasma was studied. This was estimated by measuring the release of entrapped aqueous phase marker - [^{125}I]tyraminylinulin as well as amphotericin B in the presence of buffer and plasma. The results suggest that this vesicle was unstable in retaining the entrapped aqueous phase marker. It was observed that 39.7% and 21.3% of entrapped [^{125}I]tyraminylinulin was released within 2 h in the presence of plasma and buffer respectively and by 48 h, 98.1% was released in plasma (Figure 2). However, amphotericin B did not leak out as rapidly and was associated with the vesicles (only 22.5% and 10% of the amphotericin B was released in 48 h following incubation in plasma and buffer respectively). ABCV seems to differ from some conventional liposomes which have been reported to have a high retention of aqueous phase even in the presence of plasma (20).

Plasma Pharmacokinetics of Amphotericin B Delivered Through Deoxycholate Suspension and Cholesterol Hemisuccinate Vesicles in Normal and Infected Mice

Mean plasma amphotericin B concentrations-versus-time curves after iv administration of ABCV and AmB_{DOC} to normal and infected Balb/c mice were evaluated and the pharmacokinetic values are given in Table I. As can be seen from Figure 3, ABCV is rapidly cleared from the blood (CL = 23.7 mL/h.kg) with a very short initial phase half life ($t_{1/2}$ = 0.18 h). In contrast, a slower clearance of AmB_{DOC} was observed (CL = 12.25 mL/h.kg) with an initial phase half life of 0.289 h. Consequently, the concentration of amphotericin B present in plasma 1 h after administration of AmB_{DOC} was 3-fold higher as compared to ABCV. The volume of distribution for ABCV (V_c = 765 mL/kg) was almost 3 fold higher as that of

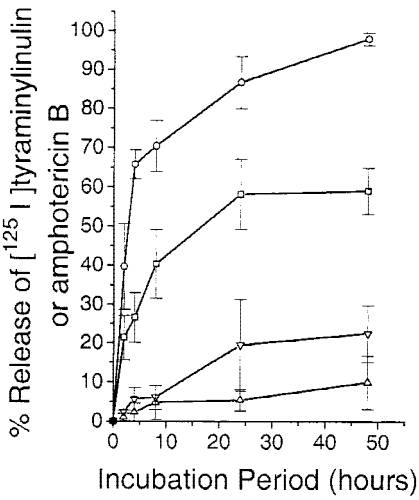

Fig. 2. Amphotericin B and [^{125}I]tyraminylinulin released from ABCV after incubation in plasma and buffer for different time intervals. Values are expressed mean of percent release of two separate experiments ± standard error of means. Release of : ○ - [^{125}I]tyraminylinulin in plasma, □- [^{125}I]tyraminylinulin in buffer, ▽ - amphotericin B in plasma, △ - amphotericin B in buffer.

Table I. Pharmacokinetics of ABCV Versus AmB_{DOC} in Plasma

Drug	State of mice	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (μg·h/mL)	V_c (mL/kg)	CL (mLh · kg)
ABCV	Normal	0.18	506.1	765	23.7
ABCV	Infected*	0.143	342.3	2220	35
AmB_{DOC}	Normal	0.289	977.6	270	12.2
AmB_{DOC}	Infected*	0.289	861.3	280	13.9

* Infected mice were treated with ABCV or AmB_{DOC} 24 h after injection of spores.

AmB_{DOC} (V_c = 270 mL/kg), again suggesting the rapid distribution of amphotericin B to various organs. The systemic exposure of amphotericin B was described by measuring the area under the plasma concentration vs time curve ($AUC_{0-\infty}$). The $AUC_{0-\infty}$ after AmB_{DOC} administration (977.6 μg.h/mL) was almost 2 fold higher than the $AUC_{0-\infty}$ of ABCV (506 μg.h/mL). This demonstrated the higher exposure of amphotericin B to the blood compartment following AmB_{DOC} administration. It was observed that the clearance of ABCV was faster in infected mice (CL = 35.05 mL/h.kg) compared to normal mice (23.7 mL/h.kg). However the clearance of AmB_{DOC} in infected mice (CL = 13.9 mL/h.kg) was similar to the clearance in normal mice (CL = 12.25 mL/h.kg). Consequently the initial phase half life of ABCV was shorter in infected mice (0.143 h) than normal mice (0.18 h) while it remained unaltered for AmB_{DOC} (0.289 h).

The increased clearance of ABCV in infected animals corresponded with an almost 3 fold higher volume of distribution (V_c = 2220 mL/kg). As expected, the volume of distribution of AmB_{DOC} was similar in normal and infected mice. The higher clearance of ABCV, increased volume of distribution and lower initial phase half life in the infected state lead to decrease in its systemic exposure ($AUC_{0-\infty}$ decreased to 342.3 μg.h/mL). However the $AUC_{0-\infty}$ values for AmB_{DOC} were similar in normal and infected mice.

Distribution of Amphotericin B in Different Tissues of Normal and *A. fumigatus* Infected Mice

The levels of amphotericin B in various tissues after administration of AmB_{DOC} and ABCD are shown in Figure 4. It was observed that the uptake of amphotericin B in liver in the case of both ABCV ($AUC_{0-\infty}$ = 5073 μg.h/mL) as well as AmB_{DOC} ($AUC_{0-\infty}$ = 1838 μg.h/mL) was more than plasma or any other organ (Table II). Uptake of amphotericin B in liver was more after ABCV administration with higher peak concentrations (C_{max} = 118 μg/g) as compared to AmB_{DOC} (C_{max} = 71.2 μg/g). It was observed that the time of peak concentrations (T_{max}) following ABCV administration was 8 h whereas amphotericin B levels started decreasing from liver 1 h after administration of AmB_{DOC} (T_{max} = 1 h). Consequently, by 48 h amphotericin B levels in the liver were 5 - fold more after ABCV administration as compared to AmB_{DOC}.

The tissue distribution pattern of amphotericin B following administration of ABCV and AmB_{DOC} in Balb/c mice infected with *A. fumigatus* was also evaluated. Following

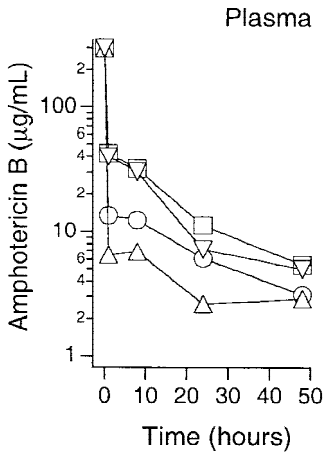

Fig. 3. Plasma amphotericin B concentration after iv administration of ABCV and AmB_{DOC} to normal and *A. fumigatus* infected Balb/c mice. Six mice at each time interval were sacrificed after administration of 12 mg/kg wt ABCV or 2 mg/kg wt AmB_{DOC} . For the purpose of comparison the AmB_{DOC} dose has been adjusted to 12 mg/kg wt Values are expressed as mean concentration of duplicate samples. ○ - ABCV (normal), □ - AmB_{DOC} (normal), △ - ABCV (infected), ▽ - AmB_{DOC} (infected).

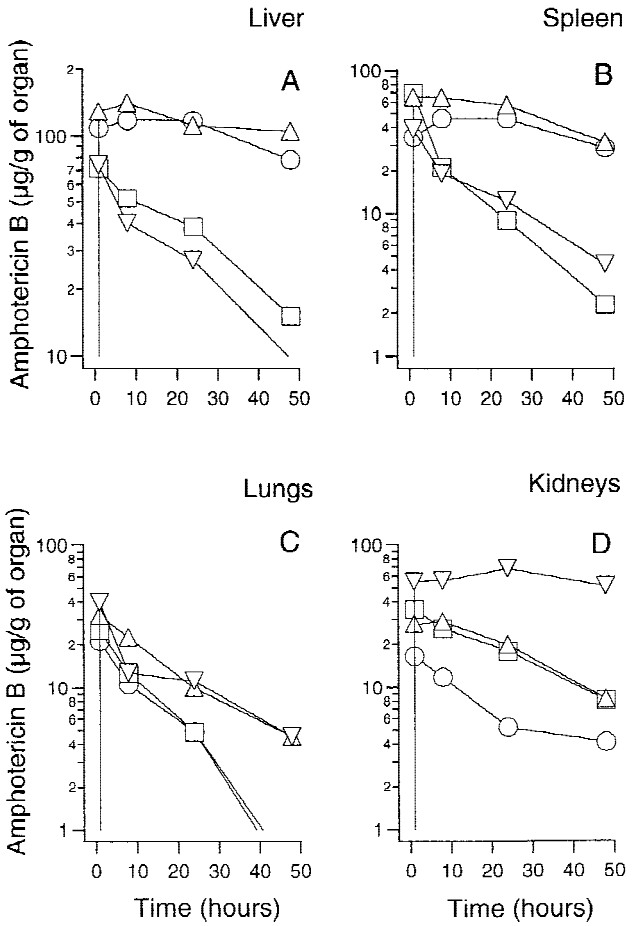

Fig. 4. (A-D). Concentrations of amphotericin B in various tissues after iv administration of ABCV and AmB_{DOC}. Other legends are same as Figure 3. At 48 h time point, due to very low concentrations of amphotericin B in some of the organs, the values cannot be seen in the figure. These values are – 9.7μg/g after AmB_{DOC} administration in the liver of infected mice; 0.4μg/g after ABCV administration in the lungs of normal mice ; 0.5μg/g after AmB_{DOC} administration in the lungs of normal mice.

Table II. Pharmacokinetic Parameters of ABCV and AmB_{DOC} in Different Tissues

		Organ											
		Liver			Lung			Spleen			Kidney		
Drug	State of mice	$AUC_{0-\infty}$ (μg·h/mL)	T_{max} (h)	C_{max} (μg/g)	$AUC_{0-\infty}$ (μg·h/mL)	T_{max} (h)	C_{max} (μg/g)	$AUC_{0-\infty}$ (μg·h/mL)	T_{max} (h)	C_{max} (μg/g)	$AUC_{0-\infty}$ (μg·h/mL)	T_{max} (h)	C_{max} (μg/g)
ABCV	Normal	5073.4	8	118	307.9	1	21.1	1937.4	8	46.1	354.1	1	16.5
ABCV	Infected*	5634.5	8	141	639.1	1	32.1	2528.1	1	64.8	937.8	8	28.8
AmB_{DOC}	Normal	1838.4	1	71.2	350.6	1	25.1	730.7	1	70.1	894	1	35.3
AmB_{DOC}	Infected*	1413.9	1	73.6	580.4	1	39.6	671.3	1	38.7	2847.4	24	68.2

* Infected mice were treated with ABCV OR AmB_{DOC} 24 h after injection of spores.

ABCV administration, uptake of amphotericin B in the liver of infected mice ($AUC_{0-\infty}$ = 5634 μg.h/mL) was found to be higher compared to normal mice ($AUC_{0-\infty}$ = 5073 μg.h/mL). The C_{max} of amphotericin B in infected mice was observed to be 141 μg/g in comparison to 118 μg/g for normal mice though the time of peak concentrations (T_{max} = 8 h) remained unaltered. In contrast, after AmB_{DOC} administration the uptake of amphotericin B in liver decreased in the infected state ($AUC_{0-\infty}$ = 1413 μg.h/mL). Though the C_{max} (73.6 μg/g) was similar to normal mice (C_{max} = 71.25 μg/g) at 1 h, the rate of disappearance of amphotericin B from the liver was faster and lower levels were obtained at 48 h in infected mice.

Though the uptake of amphotericin B in spleen 1 h after ABCV administration (3.1%) was found to be 2 fold lower compared to AmB_{DOC} administration (6.4%), the total uptake in ABCV administered mice was higher. It was observed that spleen $AUC_{0-\infty}$ for ABCV (1937.4 μg.h/g) was significantly higher than AmB_{DOC} (730.7 μg.h/g). The concentration of amphotericin B in AmB_{DOC} administered mice decreased after 1 h and by 48 h, lower amounts were present in spleen (0.2%) in comparison to ABCV administration (2.6%). The uptake of amphotericin B in spleen after ABCV administration to infected mice ($AUC_{0-\infty}$ = 2528 μg.h/mL) was found to be higher compared to normal mice ($AUC_{0-\infty}$ = 1937 μg.h/mL). The C_{max} of amphotericin B in infected mice was observed to be 64.8 μg/g in comparison to 46.1 μg/g for normal mice. In contrast, after AmB_{DOC} administration the uptake of amphotericin B in spleen decreased in infected mice ($AUC_{0-\infty}$ = 671.3 μg.h/g) as compared to normal mice ($AUC_{0-\infty}$ = 730 μg.h/g). The peak concentration in infected mice (C_{max} = 38.7 μg/g) was observed to be significantly lower than normal mice (70.1 μg/g).

High bioaccumulation could be clearly observed in the liver and spleen from the higher $AUC_{0-\infty}$ as well as the percent retention of amphotericin B over the 48 h study period. It should be noted that during multiple dose treatment with ABCV, doses would have to be carefully adjusted so that the amphotericin B levels in the tissues do not reach toxic levels, but remains in the therapeutic range.

The uptake of amphotericin B in lungs after ABCV administration ($AUC_{0-\infty}$ = 307 μg.h/g) was observed to be similar to AmB_{DOC} (350 μg.h/g). Peak concentrations were observed at 1 h (T_{max} = 1 h) for both AmB_{DOC} (C_{max} = 25.1 μg/g) as well as ABCV (C_{max} = 21.1 μg/g). After that the levels declined and by 48 h negligible amounts were present in the lungs of ABCV (0.033%) and AmB_{DOC} (0.041%) administered mice. In the infected state, increases in the uptake of amphotericin B were observed for both ABCV ($AUC_{0-\infty}$ = 639 μg.h/g; C_{max} = 32.1 μg/g) as well as AmB_{DOC} administration ($AUC_{0-\infty}$ = 580 μg.h/g; C_{max} = 39.6 μg/g).

In the kidneys, the major target organ for amphotericin B mediated toxicity, uptake of amphotericin B was more than 2 fold higher after AmB_{DOC} administration ($AUC_{0-\infty}$ = 894 μg.h/g) in comparison to ABCV ($AUC_{0-\infty}$ = 354 μg.h/g). Throughout the course of the experiment, the levels of amphotericin B remained 2–3 fold higher in kidneys after administration of AmB_{DOC} compared to ABCV. The peak concentration of amphotericin B in ABCV administered mice (C_{max} = 16.5 μg/g) was significantly lower than AmB_{DOC} treatment (35.3 μg/g). For both AmB_{DOC} and ABCV, peak concentrations were observed at 1 h (T_{max} = 1 h) after which the amphotericin B levels declined. The uptake of amphotericin B in kidneys of infected mice after both ABCV ($AUC_{0-\infty}$ = 937 μg.h/g) and AmB_{DOC} ($AUC_{0-\infty}$ = 2847 μg.h/g) administration was observed to be higher than in normal mice. Higher uptake in kidneys is expected due to damage in capillaries in the infected state and has also been explained earlier by other workers (21). However total exposure and peak concentrations of amphotericin B with AmB_{DOC} administration (C_{max} = 68.2 μg/g) were significantly higher in comparison to ABCV administration (C_{max} = 28.8 μg/g).

After administration of AmB_{DOC}, 63.8% of the total dose was recovered at 1 h and the recovered dose decreased till 48 h (10.8%). Following ABCV administration, 65.3% of the administered dose was recovered in 1 h and by 48 h, 42.4% of the amphotericin B was still recovered. In the case of infected mice, the recovery of amphotericin B increased in both ABCV and AmB_{DOC} administered mice (Tables III and IV).

DISCUSSION

Earlier we have shown that delivery of amphotericin B through cholesterol hemisuccinate vesicles significantly reduces its toxicity and increases its therapeutic efficacy in comparison to AmB_{DOC} (15). We proposed that this reduction in toxicity may be due to the rapid elimination of amphotericin B from circulation and accumulation in the liver leading to reduced levels in kidneys, the prime target organ of toxicity as reported for other lipid based formulations of amphotericin B (13,14). In this report we directly show that the pharmacokinetics of amphotericin B after delivery through sterol vesicles is significantly altered as compared to AmB_{DOC}. We observed that there was rapid elimination of amphotericin B from the plasma and accumulation mainly in the liver after administration of ABCV. Because of this accelerated removal

Table III. Tissue Distribution of Amphotericin B After ABCV Administration to Normal and *A. fumigatus* Infected Balb/c Mice Expressed as a Percent Uptake of Total Injected Dose

Time interval after administration of ABCV (h)	% Uptake											
	Plasma		Liver		Spleen		Lungs		Kidney		Total	
	N	I	N	I	N	I	N	I	N	I	N	I
1	4.5	2.1	52.9	62.6	3.1	5.9	1.7	2.6	3.1	5.2	65.3	78.4
8	4.1	2.3	57.5	68.7	4.2	5.9	0.87	1.8	2.1	5.4	68.7	84.1
24	2.0	0.87	57.1	54.4	4.2	5.2	0.40	0.82	0.97	3.7	64.6	64.9
48	1.0	0.96	38.0	51.4	2.6	2.9	0.033	0.37	0.77	1.5	42.4	57.1

Note: Six mice were sacrificed at each time interval after iv administration of 12 mg/kg wt ABCV. Infected mice were treated with ABCV 24 h after injection of spores. Normal; I-Infected.

from circulation as well as encapsulation of amphotericin B in a vesicular structure while in circulation, deleterious interactions with RBC are minimized and therefore toxicity is significantly reduced. As 57% and 38% of the injected amphotericin B was recovered from the liver after 24 and 48 h respectively, it is apparent that it is not degraded rapidly in the liver.

Therefore, like other lipid based formulations of amphotericin B, ABCV was also found to have altered pharmacokinetics in comparison to AmB_{DOC}. Like ABCV, ABLC (9,13), ABCD (10,14,22), AmBisome (23) also have been reported to have enhanced uptake in liver. Similarly, all of them had reduced levels in the kidneys, which is a significant observation, considering that amphotericin B is a nephorotoxic drug. While AmBisome has a prolonged plasma half life, all other lipid formulations were cleared rapidly from circulation.

It was observed that the time of peak concentration of amphotericin B after ABCV administration was earlier in kidneys and lungs (T_{max} = 1 h) in comparison to liver and spleen (T_{max} = 8 h). That is, the levels in liver and spleen continued to increase despite decreasing plasma concentrations. Similar observations have been recently reported for liposomes (24). It has been proposed that liposomal drugs in reticuloendothelial tissues are not in free equilibrium with plasma as are non-liposomal drugs. This may explain the high retention of ABCV in reticuloendothelial organs compared to conventional amphotericin B.

We observed that there was a significant enhancement in uptake of amphotericin B from cholesterol hemisuccinate vesicles in infected tissues like liver, spleen and kidneys compared to tissues of normal mice. It has been reported by a number of investigators that there is higher uptake of amphotericin B from lipid based formulations in infected tissues (21). The higher uptake of amphotericin B in infected organs has been attributed to damage in capillaries of various organs (21), and delivery of drugs to the infected site via circulating monocytes and macrophages which have the ability to phagocytose amphotericin B containing lipid vesicles in circulation as well as a tendency to migrate to the infected site (25). Therefore it appears that in the case of aspergillosis, circulating monocytes or macrophages may phagocytose ABCV vesicles and subsequently deliver them to infected sites thereby increasing the local concentration of drug resulting in improved therapeutic efficacy.

The amphotericin B cholesterol hemisuccinate vesicles prepared in the present investigation are significantly different in nature from the conventional liposomes used for drug delivery. Liposomes have been reported to have much higher stability with respect to aqueous entrapment (20,26). However our prime interest lies in the delivery of amphotericin B and it was found to be associated with the vesicles (only 22.5% leakage over 48 h in plasma). In the future, electron microscopy could provide us additional information on the characteristics of these vesicles, whereby we may be able to further characterize the leakage of aqueous marker and retention of amphotericin B in these vesicles.

In our previous report we demonstrated the enhanced therapeutic efficacy of ABCV against aspergillosis and in the present work we have tried to explain the mechanistic basis for reduced toxicity and enhanced therapeutic efficacy. These pharmacological studies demonstrate that altered biodistribu-

Table IV. Tissue Distribution of Amphotericin B After AmB_{DOC} Administration to Normal and *A. fumigatus* Infected Balb/c Mice Expressed as a Percent Uptake of Total Injected Dose

Time interval after administration of AmB_{DOC} (h)	% Uptake											
	Plasma		Liver		Spleen		Lungs		Kidney		Total	
	N	I	N	I	N	I	N	I	N	I	N	I
1	14.1	13.3	34.7	35.8	6.4	3.5	2.0	3.3	6.6	10.3	63.8	66.2
8	10.6	10.0	25.3	19.5	1.9	1.7	1.0	1.0	4.7	10.5	43.5	42.7
24	3.7	2.4	18.8	13.3	0.82	1.1	0.40	0.92	3.3	12.7	27.0	30.4
48	1.8	1.6	7.3	4.7	0.21	0.40	0.041	0.37	1.5	9.6	10.8	16.6

Note: Six mice were sacrificed at each time interval after iv administration of 2 mg/kg wt AmB_{DOC}. For the purpose of comparison the AmB_{DOC} dose has been adjusted to 12 mg/kg wt. Infected mice were treated with AmB_{DOC} 24 h after injection of spores. Normal; I-Infected.

tion of ABCV is the reason for reduction of toxicity and enhancement of therapeutic efficacy in comparison to AmB_{DOC}. Higher uptake of amphotericin B in liver with concurrent low levels in kidneys and plasma and enhanced localization at infected sites lead to the higher observed therapeutic efficacy of ABCV.

The cost of treatment with lipid formulations of amphotericin B has been a cause of concern in its use as a therapeutic agent. The cost of treatment is very high and constitutes a hindrance to widespread use in poor countries like ours. Considering this ABCV was prepared from cholesterol hemisuccinate which costs only $ 2/gram. Other antifungal formulations prepared from lipids like cholesterol-3-sulfate ($ 300/gram), Dimyristoyl phosphatidyl choline ($ 229/gram), Dimyristoyl phosphatidyl glycerol ($ 450/gram) and Distearoyl phosphatidyl glycerol ($ 450/gram) are much more expensive to produce. Another advantage of ABCV is its simplicity of preparation. Since the intercalation efficiency of amphotericin B in ABCV is about 90–95%, procedures like dialysis to remove free amphotericin B are not essential. Summarizing, ABCV seems to be similar to other lipid formulations in its extensive reticuloendothelial system uptake, reduction in kidney levels and increased therapeutic index all of which support its clinical evaluation as an effective but more economical alternative to present commercial formulations.

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REFERENCES

1. G. Medoff, J. Brajtburg, G. S. Kobayashi, and J. Bolard. Antifungal agents useful in therapy of systemic fungal infections. *Annu. Rev. Pharmacol. Toxicol.* **23**:303–330 (1983).
2. B. Dekruyff, W. J. Gerristen, A. Oerlemans, R. A. Demel, and L. L. M. Van Deenen. Polyene antibiotic-sterol interactions in membrane of Acholeplasma ladlawii cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by polyene antibiotics. *Biochim. Biophys. Acta* **339**:30–43 (1974).
3. D. W. Denning and D. A. Stevens. Antifungal and surgical treatment of invasive aspergillosis: Review of 2,121 published cases. *Rev. Infect. Dis.* **12**:1147–1201 (1990).
4. M. S. Maddux and S. L. Barriere. A review of complications of amphotericin B therapy: Recommendations for prevention and management. *Drug Intell. Clin. Pharm.* **14**:177–181 (1980).
5. J. W. Hiemenz and T. J. Walsh. Lipid formulations of amphotericin B: Recent progress and future directions. *Clin. Infect. Dis.* **22**:133–144 (1996).
6. R. Janknegt, S. De Marie, I. A. J. M. Bakker-Woudenberg, and D. J. A. Crommelin. Liposomal and lipid formulations of amphotericin B: Clinical pharmacokinetics. *Clin. Pharmacokinet.* **23**: 279–291 (1992).
7. S. De Marie, R. Janknegt, and I. A. J. M Bakker-Woudenberg. Clinical use of liposomal and lipid-complexed amphotericin B. *J. Antimicrob. Chemother.* **33**:907–916 (1994).
8. I. Bekersky, R. M. Fielding, D. Buell, and I. Lawrence. Lipid-based amphotericin B formulations: From animals to man. *Pharmaceut. Sci. Technol.Today.* **2**:230–236 (1999).
9. J. M. Clark, R. R. Whitney, S. J. Olsen, R. J. George, M. R. Swerdel, L. Kunselman, and D. P. Bonner. Amphotericin B lipid complex therapy of experimental fungal infections in mice. *Antimicrob. Agents Chemother.* **35**:615–621 (1991).
10. R. M. Fielding, P. C. Smith, L. H. Wang, J. Porter, and L. S. S. Guo. Comparative pharmacokinetics of amphotericin B after administration of a novel colloidal delivery system, ABCD, and a conventional formulation to rats. *Antimicrob. Agents Chemother.* **35**:1208–1211 (1991).
11. L. S. S. Guo, R. M. Fielding, and D. D. Lasic. Novel antifungal drug delivery: Stable amphotericin B—cholesteryl sulfate discs. *Int. J. Pharm.* **75**:45–54 (1991).
12. J. A. Gondal, R. P. Swartz, and A. Rahman. Therapeutic evaluation of free and liposomal encapsulated amphotericin B in the treatment of systemic candidiasis in mice. *Antimicrob. Agents Chemother.* **33**:1544–1548 (1989).
13. S. J. Olsen, M. R. Swerdel, B. Barbara, J. M. Clark, and D. P. Bonner. Tissue distribution of amphotericin B lipid complex in laboratory animals. *J. Pharm. Pharmacol.* **43**:831–835 (1991).
14. R. M. Fielding, A. W. Singer, L. H. Wang, S. Babbar, and L. S. S. Guo. Relationship of pharmacokinetics and drug distribution in tissue to increased safety of amphotericin B colloidal dispersion in dogs. *Antimicrob. Agents Chemother.* **36**:299–307 (1992).
15. S. Saxena, J. A. Khan, and P. C. Ghosh. Toxicity and therapeutic efficacy of amphotericin B delivered through cholesterol hemisuccinate vesicles in the treatment of experimental murine aspergillosis. *J. Antimicrob. Chemother.* **42**:635–642 (1998).
16. N. Munshi, K. Chakarvorty, T. K. De, and A. N. Maitra. Activity and stability studies of ultrafine nonoencapsulated catalase and penicillinase. *Colloid Polymer Science* **276**:462–472 (1995).
17. I. Nilsson-Ehle, T. T. Yoshikawa, J. E. Edwards, M. C. Schotz, and B. G Lucien. Quantitation of amphotericin B with use of high-pressure liquid chromatography. *J. Infect. Dis.* **135**:414–422 (1977).
18. J. Wartak. *Clinical Pharmacokinetics,* Prager Publishers. New York, 1983.
19. V. M. Vasandani, S. Madan, and P. C. Ghosh. *In vivo* potentiation of ricin toxicity by monensin delivered through liposomes. *Biochim. Biophys. Acta* **1116**:315–323 (1992).
20. C. Kirby, J. Clarke, and G. Gregoriadis. Effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and *in vitro*. *Biochem. J*. **186**:591–598 (1980).
21. I. Ahmad, A. K Sarkar, and B. K. Bachhawat. Mannosylated liposome-mediated delivery of amphotericin-B in the control of experimental aspergillosis in balb/c mice. *J. Clin. Biochem. Nutrition* **10**:171–179 (1991).
22. L. H. Wang, R. M. Fielding, P. C Smith, and L. S. S. Guo. Comparative tissue distribution and elimination of amphotericin B colloidal dispersion (amphocil) and fungizone after repeated dosing in rats. *Pharm. Res.* **12**:275– 283 (1995).
23. J. W. Lee, M. A. Amantea, P. A. Francis, E. E. Navarro, J. Bacher, P. A Pizzo, and T. J. Walsh. Pharmacokinetics and safety of a unilamellar liposomal formulation of amphotericin B (amBisome) in rabbits. *Antimicrob. Agents Chemother.* **38**:713–718 (1994).
24. R. M. Fielding, R. O. Lewis, and L. Moon-McDermott. Altered tissue distribution and elimination of amaikacin encapsulated in unilamellar, low-clearance liposomes (mikasome). *Pharm. Res.* **15**:1775–1781 (1998).
25. J. R. Morgan, L. A. Williams, and C. B. Howard. Technetium-labeled liposome imaging for deep-seated infection. *Br. J. Radiol.* **58**:35–39 (1985).
26. M. J.Ostro. *Liposomes,* Marcel Dekker, Inc. New York, 1983.