Safety and Toxicokinetics of Intravenous Liposomal Amphotericin B (AmBisome®) in Beagle Dogs

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Purpose. Amphotericin B (AmB) in small, unilamellar liposomes (AmBisome®) has an improved therapeutic index, and altered pharmacokinetics. The repeat-dose safety and toxicokinetic profiles of AmBisome were studied at clinically relevant doses.

Methods. Beagle dogs (5/sex/group) received intravenous AmBisome (0.25, 1, 4, 8, and 16 mg/kg/day), empty liposomes or vehicle for 30 days. AmB was determined in plasma on days 1, 14, and 30, and in tissues on day 31. Safety parameters included body weight, clinical chemistry, hematology and microscopic pathology.

Results. Seventeen of twenty animals receiving 8 and 16 mg/kg were sacrificed early due to weight loss caused by reduced food intake. Dose-dependent renal tubular nephrosis, and other effects characteristic of conventional AmB occurred at 1 mg/kg/day or higher. Although empty liposomes and AmBisome increased plasma cholesterol, no toxicities unique to AmBisome were revealed. Plasma ultrafiltrates contained no AmB. AmBisome achieved plasma levels 100-fold higher than other AmB formulations. AmBisome kinetics were non-linear, with clearance and distribution volumes decreasing with increasing dose. This, and nonlinear tissue uptake, suggest AmBisome disposition was saturable.

Conclusions. AmBisome has the same toxic effects as conventional AmB, but they appear at much higher plasma exposures. AmBisome's non-linear pharmacokinetics are not associated with increased risk, as toxicity increases linearly with dosage. Dogs tolerated AmBisome with minimal to moderate changes in renal function at doses (4 mg/kg/day) producing peak plasma concentrations of 18–94 μg/mL.

KEY WORDS: amphotericin B; liposomes; pharmacokinetics; tissue distribution; toxicity; toxicokinetics.

INTRODUCTION

Despite the appearance of newer antifungal agents including the imidazoles, amphotericin B remains the most potent broad-spectrum agent for the treatment of disseminated fungal infections. Amphotericin B is associated with a panoply of acute and chronic side effects, the most important of which is renal impairment, with reduction in glomerular filtration rate (GFR) and irreversible renal tubular damage (1,2). Due to the high incidence and severity of these effects, the dosage of conventional amphotericin B is often dictated by the degree of renal impairment, rather than by the patient's therapeutic response.

Due to limited solubility, conventional amphotericin B is formulated with sodium deoxycholate for injection. To improve the therapeutic index of amphotericin B, several alternative liposomal and lipid-based formulations were developed (3), which have demonstrated antifungal activity and reduced toxicity in animals (4–8), and clinical trials (9–11).

AmBisome is a true liposome formulation of amphotericin B, consisting of stable, small unilamellar vesicles with a diameter less than 100 nm, whose lipid bilayers are composed of phospholipids, cholesterol and amphotericin B. In animal models of fungal infection, AmBisome was as potent as conventional amphotericin B, but with markedly reduced toxicity (12–14). This amphotericin B-like efficacy profile, coupled with improved safety, was also observed in clinical trials (15–17). Although AmBisome was safer than conventional amphotericin B, breakthrough fungal infections may have occurred in up to 10% of febrile neutropenic patients treated with either formulation (16), suggesting that AmBisome dose intensification may benefit some patients with life-threatening invasive fungal infections. The pharmacokinetics of Amphotericin B formulations differ markedly, with AmBisome producing the highest plasma exposures at a given dose (18). However, the relationship between the high plasma exposures and altered tissue distribution observed after AmBisome and its toxicologic profile, especially after repeated high level dosing, is not completely understood. To support the safe administration of AmBisome at higher dose levels, we characterized the altered safety and toxicokinetic profiles of intravenous AmBisome in dogs during a 30-day repeated–dose study at five dose levels, ranging from subclinical to a 3–5 fold multiple of the recommended dose. The objectives of this study included defining the target tissues, and dose-limiting toxicity(s) of AmBisome, and investigating the toxicokinetic relationships between dose, exposure and toxic effects for this novel formulation.

MATERIALS AND METHODS

Animals

Thirty-five male and thirty-five female beagle dogs (Harlan Sprague Dawley, Inc.), ages 4 to 6 months and weighing 8.0–10.4 kg (males) and 5.0–9.2 kg (female) were randomly assigned to the seven treatment groups (5/sex/group). Animals were housed in individual cages in temperature and humidity controlled rooms, fed daily, and allowed free access to water throughout the study. Animals were acclimatized for two weeks prior to study initiation, and were subjected to a health examination prior to acceptance in the study. Animal care and use adhered to the principles in "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996).

Test and Control Articles

AmBisome (NeXstar Pharmaceuticals, Inc., San Dimas, CA) is a lyophilized liposomal formulation containing amphotericin B, hydrogenated soy phosphatidylcholine, distearoylphosphatidylglycerol, and cholesterol (approximately 50, 213,

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84, and 52 mg/vial, respectively). The formulation also contained sucrose, disodium succinate, and α-tocopherol. AmBisome was reconstituted with sterile water immediately prior to administration to yield an isotonic preparation containing 4 mg/ml amphotericin B and 28 mg/ml total lipids in liposomes with a median diameter < 100 nm. This solution was diluted with 5% dextrose solution so that a similar volume (6 ml/kg) was administered to each group. Empty liposomes with the same lipid composition as AmBisome, but which contained no amphotericin B (NeXstar Pharmaceuticals, Inc., San Dimas, CA) were supplied as an aqueous suspension (total lipid concentration, 30 mg/ml).

Study Design

Each day for 30 consecutive days, animals received a single 5 minute intravenous infusion (cephalic vein) of AmBisome (0.25, 1, 4, 8, or 16 mg/kg based on previous days body weight), 5% dextrose solution, or empty liposomes (lipid dose equal to that of the 16 mg/kg AmBisome group). On days 1, 14, and 30 blood samples (4 mL) were obtained at 0.5, 1, 3, 5, 8, and 24 hours post dose for toxicokinetic evaluation of amphotericin B concentrations. Plasma was separated by centrifugation and approximately half was frozen at −20°C until assayed. The remaining plasma was subjected to centrifugal ultrafiltration (Centrifree®, Amicon, Inc., Beverly, MA) to obtain a protein-free ultrafiltrate which was frozen at −20°C until assayed.

Safety Evaluation

Animals were observed twice daily for signs of toxicity. Body weights were recorded the day before dosing began, on each dosing day, and immediately prior to sacrifice. Blood samples for clinical chemistry and hematologic evaluation were obtained from a jugular vein prior to initial dosing, on days 2, 3, 6, 13, 21, and 29, and from moribund animals. Overnight urine samples were collected on days 2, 3, 6, 13, 21, and 29 for urinalysis. One day following the final dose, all surviving animals were sacrificed by exsanguination under sodium pentobarbital anesthesia. Necropsy was performed on all animals, at which time samples of brain, kidney, liver, lung, and spleen were collected, blotted dry, weighed then frozen (−70°C) for amphotericin B analysis. Samples of these organs and additional selected tissues were collected and fixed in 10% phosphate buffered formalin. Tissue sections were stained with hematoxylin and eosin and examined microscopically. Observed histomorphologic changes were graded for severity on a five-point scale (minimal, slight, moderate, marked, severe).

Statistical Analysis

The statistical significance of observed differences in safety parameters between groups was evaluated using one-way ANOVA, with pairwise comparisons between treated and control groups performed using the Games and Howell Modified Tukey-Kramer test. Some data sets were transformed to achieve homogeneous variance (Levene's test).

Amphotericin B Assay

Amphotericin B concentrations were determined in plasma and plasma ultrafiltrates as previously described (19). Briefly, samples (250 μl) were deproteinized with methanol (750 μl) and the supernatants (75 μl) injected onto an Ultrasphere ODS, 250 mm × 4.6 mm i.d., 5 μm HPLC column (Beckman Instruments, Fullerton, CA). Mobile phase (methanol:acetonitrile:2.5 m*M* disodium ethylenediaminetetraacetic acid; 50:25:30) was delivered at 1 ml/min and monitored for absorption at 405 nm. The linear range in plasma was 0.05 to 20 μg/ml (correlation coefficients >0.9987) and the LOQ was 0.05 μg/ml (RSD, 5.9%). Quality control samples (0.15, 0.75 and 5.0; μg/ml) were within 4.8% of theoretical, and the absolute recovery was 75.5%. Plasma amphotericin B concentrations were calculated from linear regression calibration curves of external standard peak areas. Unknown samples were diluted as required to fall within the assay's linear range.

For tissue samples, weighed aliquots (approximately 0.5 g) of blank or sample tissues were homogenized with methanol (4.5 ml) and 10 m*M* phosphate buffer (0.5 ml), vortexed for 5 minutes, then centrifuged to obtain a clear supernatant which was injected (120 μl) into a C-18, 300 mm × 3.9 mm i.d., 10 μm HPLC column (Whatman Inc., Clifton, NJ) maintained at 35.5°C. The mobile phase (acetonitrile: 10 m*M* sodium acetate; 39.4:60.6) was delivered at 1 ml/min and monitored for absorption at 382 nm. Tissue concentrations of amphotericin B were calculated using the natural logarithm transformation of a quadratic regression equation (ln peak area = ln A + B* ln[concentration] + C*ln[concentration]^2) of amphotericin B peak areas and external standard quantitation. The linear range in tissues was 0.50 to 500.0 μg/g (correlation coefficients >0.9950) and the LOQ was 0.50 μg/g (RSD, 4.5%). Quality control samples (0.50 to 500.0 μg/g) had a RSD of ≤4.9%, and a recovery of 93.7–102.8%. Methanolic tissue extracts were diluted as required to fall within the assay's linear range.

Pharmacokinetic Analysis

Amphotericin B plasma concentration versus time data were analyzed using non-compartmental pharmacokinetic methods. The area under the plasma concentration versus time curve (AUC), the area under the first moment curve (AUMC), and the terminal elimination half-life ($t_{1/2}$) were calculated using a non-linear, least-squares curve fitting program (RSTRIP, MicroMath, Salt Lake City, UT). The AUC and AUMC from 0–24 hours were calculated using the linear trapezoidal method. The extrapolated AUC from 24 hours to infinity was calculated as C_{24hr}/β_1, and the $t_{1/2}$ was calculated as $0.693/\beta$ where β (the terminal phase elimination rate constant) was the negative slope of natural log-linear terminal portion of the plasma concentration versus time curve. Total body clearance (CL = dose/$AUC_{0-\infty}$), mean residence time (MRT = $AUMC_{0-\infty}/AUC_{0-\infty}$), volume of distribution at steady state (V_{ss} = CL × MRT), and volume of distribution (V_d = dose [$\beta \times AUC_{0-\infty}$]) were calculated using these values.

RESULTS

Clinical Observations and Necropsy

No direct drug-related deaths occurred during the study. However, all animals in the 16 mg/kg AmBisome group, and 3 of 5 males and 4 of 5 females in the 8 mg/kg AmBisome group were sacrificed prematurely, due to a loss of 25% or

more of their initial body weight. Males and females in the two control groups gained weight at similar rates. There were no significant differences between body weights in the 0.25 mg/kg and 1 mg/kg AmBisome groups compared to either control ($P > 0.05$). After 4 mg/kg AmBisome, male body weights remained unchanged while females gained weight, though less rapidly than females in lower dose groups. Body weights decreased significantly from day 3 onwards in the 8 mg/kg and 16 mg/kg AmBisome groups ($P < 0.05$ versus controls). By the second week, daily food consumption in these two groups had decreased to less than 15% of pre-study values. Although vomiting and anorexia were observed in the liposomal control group, these symptoms were more pronounced in the 4, 8, and 16 mg/kg per day treatment groups. Increases in organ weight to body weight rations ($P < 0.05$ versus controls) were observed for spleen (males and females), kidney (males and females), and liver (males) at AmBisome doses of 4 mg/kg and 8 mg/kg.

Microscopic Pathology

Histopathologic changes related to AmBisome administration were limited to the kidneys (tubular nephrosis) and tissue macrophages (Kupffer cell vacuolization with cytoplasmic inclusions, and vacuolated macrophages in spleen, bone marrow and lymph nodes). These changes were observed in the 1 mg/kg and higher AmBisome groups. Renal tubular nephrosis was characterized by tubular regeneration, tubular dilation, lymphohistiocytic infiltration, nephrocalcinosis, and tubular vacuolization and necrosis. These dose-dependent renal changes were generally minimal or absent in the 1 mg/kg group, and reached only moderate severity in the 4 mg/kg group. Tubular necrosis was observed only at the 16 mg/kg dose level. Secondary histomorphologic changes observed in some treated animals (including lymphoid depletion, hepatic lipid infiltration, muscle atrophy and gastrointestinal hemorrhage, congestion, and inflammation) were attributed to uremia associated with renal tubular changes and inanition resulting from decreased food intake.

Clinical Chemistry, Hematology, and Urinalysis

Dose-dependent increases in urea nitrogen and creatinine levels were observed in both sexes after AmBisome exposure (Table I). These changes generally increased with time over the course of the study, reaching clinically significant levels by the end of the study in the 4 mg/kg and higher AmBisome groups. Decreases in serum potassium and platelets, and increases in fibrinogen and aspartate aminotransferase were observed in some groups and timepoints, but by the end of the study these changes failed to achieve a level of statistical or clinical significance to be considered treatment—related toxic changes. No changes in alanine aminotransferase levels were observed in any group. Serum cholesterol levels at the end of the study were increased ($P < 0.05$ versus dextrose control) in the empty liposome controls and the 4 mg/kg and higher AmBisome groups. Clinical pathology data from animals sacrificed prematurely (days 5 to 28), were similar to those in animals sacrificed at the termination of the study (day 31). AmBisome at doses of 1 mg/kg or higher was associated with increased urine volume and lower urine specific gravity in both sexes ($P < 0.05$ versus controls). All other clinical chemistry, hematology and urinalysis parameters remained within normal ranges during the study, with no indication of statistically or clinically significant treatment-related changes.

Plasma Pharmacokinetics

Plasma amphotericin B concentrations declined monoexponentially, with half-lives between 5 and 20 hours in the 1,

Table I. Mean (±SD) Clinical Chemistry and Hematologic Values in Beagle Dogs (5/sex/group) After IV AmBisome

Treatment group	Sample time	Urea nitrogen (mg/dL)		Creatinine (mg/dL)		AST (U/L)		Cholesterol (mg/dL)	
		Male	Female	Male	Female	Male	Female	Male	Female
Empty liposomes	Pre-dose	13 ± 2.6	16 ± 2.4	0.7 ± 0.1	0.8 ± 0	31 ± 3	30 ± 4	217 ± 41	195 ± 17
	Day 29	14 ± 2	17 ± 2	0.9 ± 0	0.9 ± 0.1	39 ± 9	33 ± 5	313 ± 33[A]	289 ± 38[A]
Dextrose	Pre-dose	12 ± 1.1	13 ± 3	0.7 ± 0.1	0.7 ± 0.1	28 ± 4	30 ± 4	204 ± 33	193 ± 29
	Day 29	14 ± 2	14 ± 3	0.8 ± 0.1	0.8 ± 0.1	36 ± 7	32 ± 3	167 ± 26	156 ± 30
AmBisome 0.25	Pre-dose	13 ± 1.9	14 ± 3	0.7 ± 0	0.7 ± 0.1	34 ± 9	33 ± 6	200 ± 24	178 ± 33
	Day 29	17 ± 4	20 ± 4	0.9 ± 0.1	0.9 ± 0.1	36 ± 3	40 ± 4	157 ± 23[B]	147 ± 29[B]
Ambisome 1	Pre-dose	14 ± 2.9	14 ± 3	0.7 ± 0.1	0.7 ± 0.1	33 ± 6	31 ± 6	203 ± 32	180 ± 10
	Day 29	21 ± 4	24 ± 2[AB]	1.0 ± 0.1	1.0 ± 0.1	42 ± 6	41 ± 7	172 ± 22[B]	163 ± 17[B]
Ambisome 4	Pre-dose	12 ± 1.7	14 ± 2	0.7 ± 0.1	0.8 ± 0.1	31 ± 4	26 ± 4	200 ± 27	217 ± 29
	Day 29[a]	58 ± 12[AB]	52 ± 9[AB]	2.3 ± 0.4[AB]	1.9 ± 0.5[A]	48 ± 9	41 ± 5	242 ± 36[AB]	223 ± 21
AmBisome 8	Pre-dose	13 ± 1.3	13 ± 3	0.8 ± 0.1	0.7 ± 0	32 ± 3	29 ± 2	220 ± 37	194 ± 26
	Day 29[b]	62 ± 6[AB]	56	3.0 ± 0.6[AB]	3.1	54 ± 13	64	295 ± 75	258
	Termination[c]	(104 ± 7)	(86 ± 9)	(4.1 ± 0.9)	(2.7 ± 0.6)	(54 ± 17)	(56 ± 15)	ND	ND
AmBisome 16	Pre-dose	12 ± 1.8	13 ± 2	0.7 ± 0.1	0.7 ± 0.1	31 ± 6	29 ± 4	190 ± 19	199 ± 16
	Termination[d]	(212 ± 71)	(129 ± 38)	(6.8 ± 2.4)	(3.7 ± 1.3)	(143 ± 167)	(58 ± 11)	ND	ND

Note: ND, Parameter not determined due to lack of sample; AST, aspartate aminotransferase; A, significantly ($P < 0.05$) different from dextrose control group; B, significantly ($P < 0.05$) different from empty liposome control group.
[a] n = 5 males, 4 females (one female inadvertently sacrificed prematurely).
[b] n = 2 males, 1 female surviving at end of study.
[c] n = 3 males (sacrificed on Days 16, 21, 28); n = 4 females (sacrificed Days 19, 20, 25, 25).
[d] n = 5 males (sacrificed on Days 5, 15, 18, 20, 26); n = 5 females (sacrificed on Days 13, 14, 15, 18, 19).

4, 8, and 16 mg/kg AmBisome groups (Fig. 1, Table II). In the 0.25 mg/kg AmBisome group, 24 hr plasma levels were below detection, so half-lives were estimated from 0–8 hr data only. Although interindividual variation in plasma amphotericin B concentrations was observed (c.v.'s between 20–50% for all groups and times), there was no systematic difference in plasma concentrations between males and females in this study. For this reason, summary pharmacokinetic data included both sexes (Table II).

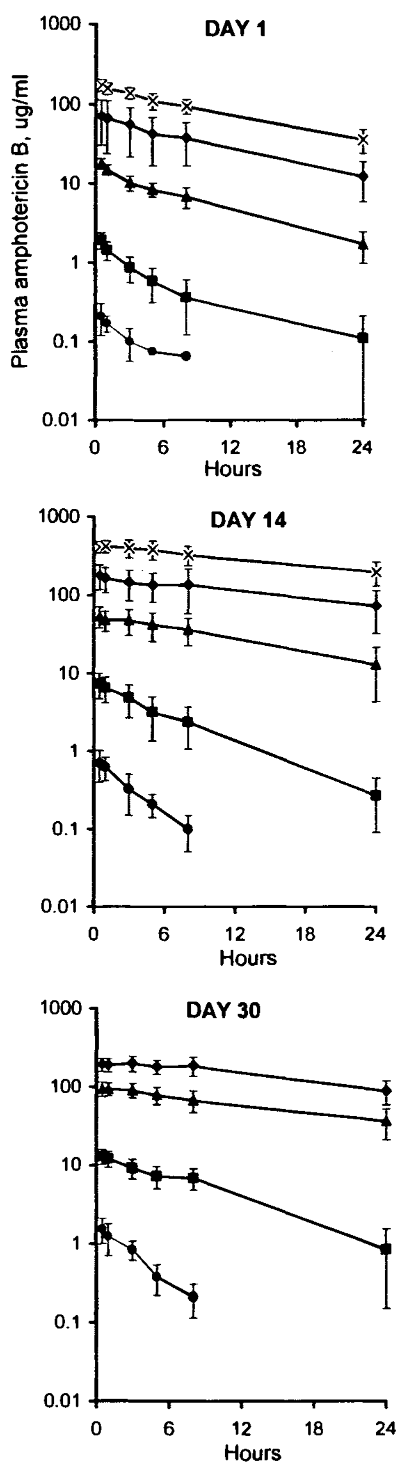

Fig. 1. Mean (±SD) plasma concentrations of amphotericin B following daily intravenous administration of AmBisome at 0.25 mg/kg (circles), 1 mg/kg (squares), 4 mg/kg (triangles), 8 mg/kg (diamonds) and 16 mg/kg (X's). In the 0.25 mg/kg group, median concentrations at 24 hr were below detection (<0.05 μg/ml) on all three study days.

Mean plasma concentrations of amphotericin B on the first day increased more than proportionally with dose (Fig. 1). Maximum plasma concentrations at 0.5 hour post infusion ranged from 0.21 ± 0.09 μg/ml at 0.25 mg/kg to 174 ± 28.5 μg/ml at 16 mg/kg, an 800-fold increase over the 64-fold dose range. Similar patterns were observed on days 14 and 30 (Fig. 1).

Plasma concentrations within each dose group also increased with multiple dosing from day 1 through day 30 (Figs. 1 and 2). Plasma concentrations of amphotericin B at 0.5 hours post infusion following 30 days of administration ranged from 1.55 ± 0.55 μg/ml for the 0.25 mg/kg per day dose to 192 ± 35 μg/ml for the 8 mg/kg per day dose (124-fold increase for a 32-fold increase in dose). The proportion by which plasma concentrations increased from day 1 through day 30 decreased as the dose level increased. Thus, only in the 8 mg/kg group did plasma concentrations approach steady-state during this 30-day study (Fig. 2).

Calculated mean pharmacokinetic parameters are shown in Table II. Marked differences were noted in these parameters between dose levels and between the first dose and subsequent doses at each dose level. For example, the day 1 AUC_{0-24} increased 1300 times over the 0.25–16 mg/kg dose range, demonstrating nonlinear plasma exposure. On Day 1, volumes of distribution (V_d and V_{ss}) decreased 12 to 23-fold over the dose range. Clearance decreased with increasing dose and number of doses. After repeated administration, $t_{1/2}$ increased 7-fold on day 14 as the dose increased from 0.25 to 16 mg/kg per day (day 30 data not available for 16 mg/kg group). However, within any given dose level, there was no clear pattern of change in $t_{1/2}$ on repeated dosing. After repeated dosing, the magnitude of the dose-related AUC_{0-24} increase was almost double (2500 times) the increase seen with a single dose. Both V_d and V_{ss} decreased within each dose group over the course of the study, approaching values close to the plasma volume as dose and time increased. Clearance also decreased over time, and with increasing dose, approaching clearances similar to those of "low-clearance" liposomes (20). The observed changes in clearance and volume of distribution are plotted as a function of the total administered dose in Fig. 3.

Protein-free plasma ultrafiltrates were analyzed from 25% of the animals administered 16 mg/kg AmBisome. They contained no detectable unbound or non-liposomal amphotericin B. Ultrafiltrates from lower dose groups were not analyzed, since it was considered unlikely they contained measurable amounts of amphotericin B.

Amphotericin B Concentrations in Tissue

Concentrations of amphotericin B in selected tissues following the 30-day study are presented in Table III. The highest concentrations were found in tissues of the reticuloendothelial system (spleen and liver). Concentrations in the kidneys were intermediate, with lowest concentrations in the lungs and brain. Although concentrations in plasma increased more than proportionally with dose, amphotericin B concentrations in the kidneys

Table II. Pharmacokinetic Parameters of Amphotericin B in Beagle Dogs (n = 10) Following IV Administration of AmBisome

Dose (mg kg⁻¹ day⁻¹)	Study day	$t_{1/2}$ (h)	MRT (h)	AUC_{0-24} (μg hr ml⁻¹)	$AUC_{0-\infty}$ (μg hr ml⁻¹)	V_d (ml/kg)	V_{ss} (ml/kg)	CL (ml hr⁻¹ kg⁻¹)
0.25	1[a]	4.6 ± 2.0[d]	23 ± 26	1.7 ± 0.4	2.6 ± 1.3	1277 ± 556	1663 ± 1352	112 ± 43
	14[b]	2.8 ± 0.8[d]	4.9 ± 3.3	2.8 ± 0.8	3.2 ± 1.0	434 ± 226	385 ± 188	77 ± 41
	30	2.5 ± 0.8[d]	4.4 ± 11	5.0 ± 1.3	5.8 ± 1.6	160 ± 44	196 ± 62	47 ± 16
1	1	8.3 ± 4.5	11.0 ± 10.7	9.5 ± 4.0	11.4 ± 4.3	1106 ± 489	959 ± 813	79 ± 44
	14	5.5 ± 1.6	6.2 ± 1.7	53 ± 26	55 ± 27	264 ± 162	140 ± 72	24 ± 14
	30	6.0 ± 1.7	7.6 ± 2.5	109 ± 40	119 ± 45	77 ± 19	67 ± 18	8.7 ± 4
4	1	8.4 ± 2.2	11.3 ± 3.6	143 ± 34	164 ± 46	302 ± 45	286 ± 85	26 ± 8
	14	9.1 ± 3.1	15.7 ± 6.0	728 ± 278	923 ± 442	65 ± 26	76 ± 26	5.2 ± 2
	30	16.0 ± 4.7	22.9 ± 6.4	1452 ± 423	3040 ± 2999	35 ± 17	37 ± 12	1.8 ± 1
8	1	11.0 ± 2.9	14.9 ± 3.9	774 ± 443	986 ± 592	144 ± 35	138 ± 38	10 ± 4
	14	18.8 ± 7.0	27.7 ± 8.8	2773 ± 1409	4980 ± 2563	54 ± 21	56 ± 24	2.4 ± 2
	30[c]	12.3 ± 7.0	22.6 ± 6.4	3598 ± 908	5320 ± 2221	26 ± 6	35 ± 8	1.7 ± 0.7
16	1	11.6 ± 3.9	15.4 ± 6.1	2277 ± 1038	2595 ± 704	105 ± 30	73 ± 47	6.5 ± 2
	14	19.8 ± 4.3	29.3 ± 6.1	7069 ± 1895	13003 ± 4647	38 ± 12	39 ± 12	1.4 ± 0.7
	30	NA	NA	NA	NA	NA	NA	NA

Note: NA, Data not available (no animals survived to Day 30 in this group).
[a] n = 5; insufficient data for parameter calculation in other animals due to concentrations below detection (<0.05 μg/ml).
[b] n = 8; insufficient data for parameter calculation in other animals due to concentrations below detection (<0.05 μg/ml).
[c] n = 3; surviving animals at Day 30).
[d] Half-lives calculated from 0–8 hour data only, median plasma concentrations at 24 hr were below detection (<0.05 μg/ml).

appeared to increase linearly with dose. In the spleen, amphotericin B concentrations increased less than dose proportionally. Liver concentrations in the 4 and 8 mg/kg groups were unavailable due to the inadvertent destruction of these samples prior to analysis, but liver concentrations were similar to those in the spleen in the 0.25 and 1 mg/kg AmBisome groups. Concentrations in tissues from animals sacrificed prematurely were similar to those in animals sacrificed at the end of the study (data not shown).

DISCUSSION

The subchronic safety and toxicokinetic profiles of intravenous AmBisome were evaluated in beagle dogs after daily doses of 0.25 to 16 mg/kg for 30 days. While no direct treatment-related deaths occurred during this study, 17 of the 20 animals exposed to AmBisome at 8 and 16 mg/kg were sacrificed early due to severe body weight loss. The observed decreases in body weight were dose dependent, and were accompanied by increases in relative organ weights, indicating these changes were secondary to a state of inanition, which resulted from a drug-induced reduction in food intake. The pattern of body weight loss in the 8 and 16 mg/kg/day AmBisome groups was similar to that reported in dogs receiving 0.75 mg/kg amphotericin B deoxycholate every other day (21).

Renal pathologic changes observed after AmBisome at 4 mg/kg/day or higher, characterized by tubular vacuolization and nephrosis, uremia, and elevated urea nitrogen and creatinine, were similar to those reported in dogs exposed to amphotericin B deoxycholate (0.75 mg/kg on alternate days) (21). Other effects observed in our study, including decreased urine specific gravity, were also reported after administration of amphotericin B deoxycholate to dogs (0.6 mg/kg per day) (4). Males appeared to be more sensitive to AmBisome than females in this study, as observed for amphotericin B deoxycholate (4). Similar renal and hepatic toxicities were reported in dogs administered 5 mg/kg per day of amphotericin B cholesteryl sulfate (AMPHOTEC®) for 14 days (4). Thus, all of the toxic effects noted with subchronic administration of AmBisome have been observed for other amphotericin B formulations.

In addition to the pathologic changes noted above, repeated dosing with AmBisome resulted in lipid accumulation in the body, as evidenced by increased plasma cholesterol levels and vacuolization of Kupffer cells and other tissue macrophages. Similar changes have been reported after intravenous administration of other liposomes, and dogs reportedly tolerated subchronic administration of liposomal lipid doses higher than those used in this study (20). There is currently no evidence suggesting that these lipid changes are associated with any significant toxicologic effects.

AmBisome produced plasma concentrations markedly higher than other amphotericin B formulations. In dogs, maximally tolerated doses of AMB (0.6–0.75 mg/kg) produced peak amphotericin B concentrations of 1.25 to 4.4 μg/ml following multiple dose administration (4,21). Daily administration of 2.5 mg/kg of amphotericin B cholesteryl sulfate for 14 days yielded a mean peak amphotericin B concentration of 1.07 μg/ml (4). After 14 days of AmBisome at 4 mg/kg per day, the mean peak (0.5 hr) amphotericin B plasma concentration was 54 ± 16 μg/ml.

The disposition of AmBisome was markedly nonlinear, with plasma concentrations and AUC_{0-24} increasing with multiple dosing. Nonlinear kinetics have also been reported with other formulations of amphotericin B. For example, after 14 days treatment with amphotericin B cholesteryl sulfate (2.5 mg/kg/day), plasma amphotericin B peak concentration decreased by 30% while trough concentration increased by 230% (4).

Although the day 14 AUC_{0-24} for AmBisome increased 2500 times over the 64-fold dose range, the $t_{1/2}$ increased only

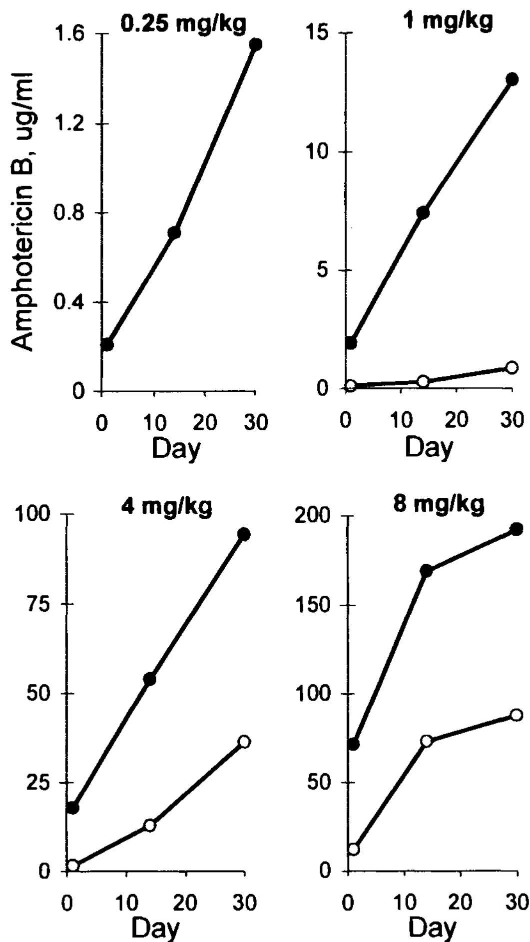

Fig. 2. Mean peak (filled circles) and trough (open circles) concentrations of amphotericin B in plasma after daily intravenous AmBisome. Peak levels were measured 0.5 hr after dosing, trough levels 24 hr after dosing. In the 0.25 mg/kg group, median concentrations at 24 hr were below detection (<0.05 μg/ml) on all three study days.

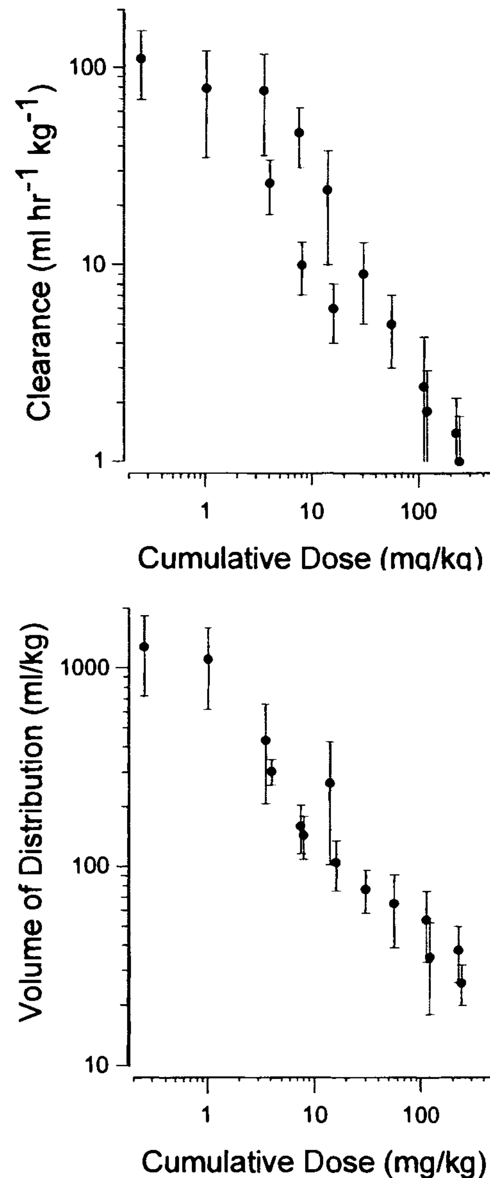

Fig. 3. The relationship between changes in the intrinsic pharmacokinetic parameters (clearance and volume of distribution) and the total administered dose of intravenous AmBisome. Values shown are group means (±SD).

7-fold, suggesting that the 55-fold decrease in clearance was primarily attributed to the decreasing volume of distribution. Adler-Moore and Proffitt (22) reported AmBisome was stable for 72 hours in the presence of plasma and red blood cells and that intact AmBisome localized at sites of *Candida* infection in mice. Decreases in volumes of distribution could reflect saturable uptake of intact AmBisome by the reticulo-endothelial system (RES) at higher doses, or the transfer of drug from liposomes into tissues. The decrease in volume with dose is also consistent with the higher, but apparently saturable levels of amphotericin B we observed in the RES compared to other tissues (Table III). Because the analytical procedure did not distinguish liposomal from non-liposomal amphotericin B, direct evidence for intact AmBisome uptake in these organs is lacking. However, analysis of plasma ultrafiltrates demonstrated there was no detectable non-liposomal amphotericin B in the plasma. As noted above, plasma concentrations after amphotericin B deoxycholate dosing are much lower than with AmBisome administration.

Amphotericin B deoxycholate doses as low as 1 mg/kg on alternate days in dogs resulted in the death of all animals (21), while dogs tolerated daily AmBisome at 4 mg/kg with only moderate renal toxicity. Given the toxicity of amphotericin B deoxycholate at much lower plasma exposures, it seems unlikely that the animals in this study would have survived if a significant fraction of the amphotericin B in plasma and tissue were non-liposomal.

In this subchronic study, dogs tolerated plasma concentrations of amphotericin B approximately 2 orders of magnitude greater than could safely be achieved with other formulations. The dose-limiting toxicities of AmBisome seen at these elevated concentrations were qualitatively and quantitatively similar to those seen at lower exposures to other amphotericin B formulations, with no additional target tissues identified. The nonlinear disposition of AmBisome in dogs resulted in a more than proportional increase in AUC_{0-24}, while clearance and volumes of distribution decreased in proportion to the total administered

Table III. Mean (±SD) Tissue Concentrations (μg/g) of Amphotericin B in Beagle Dogs (n = 10) One Day After the Last of 30 Consecutive Daily Doses of AmBisome

	AmBisome dose (mg kg^{-1} day^{-1})			
Tissue	0.25	1	4	8
Liver	93.8 ± 15.4	314.8 ± 40[a]	NA	NA
Kidneys	1.39 ± 0.72	15.1 ± 4.2	73.6 ± 13[a]	101.4 ± 4.9[c]
Spleen	116.5 ± 31.1	295.3 ± 71.9	765 ± 113[a]	1065 ± 109[c]
Lungs	0.11 ± 0.33	3.0 ± 1.5[b]	31.2 ± 8.1[a]	47.3 ± 8.8[c]
Brain	BLOQ	BLOQ	0.34 ± 0.33[b]	1.54[d]

Note: NA, Data not available (liver samples from the 4 and 8 mg/kg dose groups were inadvertently destroyed prior to analysis); BLOQ, below assay limit of quantitation (< 0.5 μg/g)
[a] n = 9; one female sacrificed prematurely.
[b] n = 7; two of the nine brain samples destroyed prior to analysis.
[c] n = 3; three males and four females sacrificed prematurely.
[d] n = 1; two of the three brain samples destroyed prior to analysis.

dose (Fig. 3). However, this nonlinearity did not result in increased toxic risk. Quantitative measures of toxicity rose, along with kidney drug concentration, in a relatively linear fashion with increasing dose (Fig. 4). Thus, renal amphotericin B concentration appears to be a better predictor of AmBisome toxicity than plasma exposure. It is interesting to note that, despite the marked differences in pharmacokinetics between AmBisome, amphotericin B deoxycholate and amphotericin B cholesteryl sulfate (18), the magnitude of renal toxicity correlates well with total concentrations of amphotericin B in the kidneys (Fig. 4), while increased levels of amphotericin B in the liver from the lipid formulations are not associated with hepatotoxicity. Dose-limiting renal toxicity was observed for all three formulations as amphotericin B concentrations in the kidneys rose to above 80–100 μg/g (Table III, ref. 4). However, AmBisome achieved a plasma amphotericin B exposure (day 14 AUC_{0-24}) that was 50 to 100-fold higher than the other two formulations.

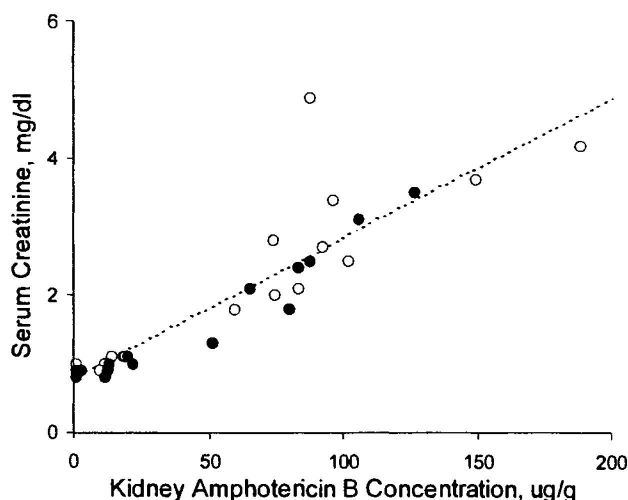

Fig. 4. The relationship between day 29 serum creatinine, a quantitative measure of renal toxicity, and the concentration of amphotericin B in the kidneys after 30 daily injections of AmBisome. Data points are from individual animals (males, open circles, females, closed circles). Dotted line shows linear regression fit (r^2 = 0.83).

Similar nonlinear pharmacokinetics were observed for AmBisome in rats and humans (15,23). However, the magnitude of these changes was larger in the dog. Maximum volumes of distribution observed in the dog were several-fold higher than those in other species, and approached the volume of distribution of conventional amphotericin B (>1000 ml/kg). As the total administered dose increased, the volume of distribution fell to values close to that of the plasma compartment (≤40 ml/kg). In a similar manner, AmBisome clearance fell from values at or above the clearance of conventional amphotericin B (>100 ml hr^{-1} kg^{-1}), to values (approx. 1 ml hr^{-1} kg^{-1}) close to those of "long-circulating" liposomes (20). One possible explanation for these results is that at higher cumulative doses, a larger fraction of the administered drug remains within long-circulating liposomes in the plasma, due to the presence of a rapid but saturable clearance mechanism, as previously described for RES clearance of liposomes (24). Since amphotericin B in circulating liposomes is likely to be less toxic than amphotericin B bound to lipoproteins or cells, the relatively linear increase in kidney amphotericin B concentration and toxic response with dose could be explained even in the presence of a much more rapidly increasing total plasma exposure if an increasing fraction of the amphotericin B in plasma is sequestered within the liposomes as the dose increases.

CONCLUSIONS

Although nonlinear kinetics resulted in more-than-proportional increases in amphotericin B plasma exposure with dose and time, AmBisome was tolerated at plasma exposures many times those of other amphotericin B formulations. The dose-limiting renal toxicity observed with AmBisome was dose proportional and similar in spectrum to conventional amphotericin B, with no toxic endpoints unique to this formulation. Although dogs are more sensitive to amphotericin B than other species, they tolerated AmBisome with minimal to moderate changes in renal function at a dose regimen (4 mg/kg/day) producing peak plasma concentrations of 18–94 μg/ml over the course of the 30-day study.

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