CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER: 050740

MICROBIOLOGY REVIEW(S)

REVIEW FOR HFD-530
OFFICE OF NEW DRUG CHEMISTRY
MICROBIOLOGY STAFF
MICROBIOLOGIST'S REVIEW #1 OF NDA

APR 19 1997

18 April 1997

A. 1. NDA 50-740

SPONSOR Fujisawa USA
Deerfield, Illinois

2. PRODUCT NAMES: AmBisome® for Injection (liposomal amphotericin B)

3. DOSAGE FORM AND ROUTE OF ADMINISTRATION: Lyophilized liposomes containing Amphotericin B in a 30 cc vial for intravenous injection

4. METHOD(S) OF STERILIZATION:

5. PHARMACOLOGICAL CATEGORY: Anti-fungal

6. DRUG PRIORITY CLASSIFICATION: 3P

B. 1. DATE OF INITIAL SUBMISSION: 8 November 1996

2. DATE OF AMENDMENT: (none for consult review)

3. RELATED DOCUMENTS:

4. ASSIGNED FOR REVIEW: 19 December 1997

C. REMARKS: The product is manufactured for Fujisawa USA by NeXstar

D. CONCLUSIONS: The application is not recommended for approval for reasons of sterility assurance.

/S/ 4-18-97

David Hussong, Ph.D.

4/19/97

/S/

cc:

HFD 160/Consult File
HFD 530/CSO
HFD 830/Chemist/N. Schmuff
HFD 805/Consult File
HFD 805/D. Hussong

APPEARS THIS WAY
ON ORIGINAL

Drafted by: D. Hussong, 4/14/97
R/D initialed by: P. Cooney

Filename, c:\d\nda\50-740r1.wpd

MICROBIOLOGY REVIEW
DIVISION OF SPECIAL PATHOGENS AND IMMUNOLOGIC DRUG PRODUCTS (HFD-590)

NDA #: 50-740

REVIEWER : Shukal Bala
CORRESPONDENCE DATE : 06-21-96; 11-08-96
06-13-97
CDER RECEIPT DATE : 06-25-96; 11-12-96
06-16-97
REVIEW ASSIGN DATE : 06-28-96; 11-15-96
06-19-97
REVIEW COMPLETE DATE : 06-30-97

SPONSOR: Fujisawa Pharmaceutical Company
Three Parkway North
Parkway North Center, 4th Floor
Deerfield, IL 60015

SUBMISSION REVIEWED: Pre-submission, Original and BI

DRUG CATEGORY: Anti-fungal/anti-parasitic

INDICATION: Treatment and prophylaxis of systemic fungal infections; empiric therapy for presumed fungal infections; treatment of visceral leishmaniasis

DOSAGE FORM: Liposomal formulation for systemic injection

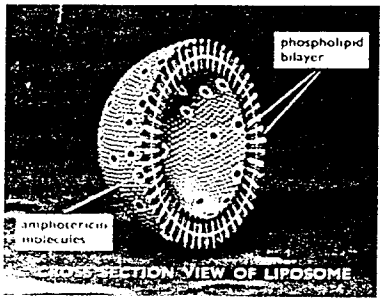
PRODUCT NAMES:

a. **PROPRIETARY:** AmBisome^R

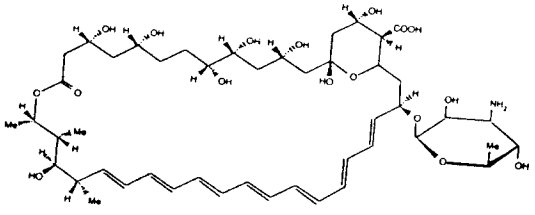
b. **NONPROPRIETARY:** VS104

c. **CHEMICAL:** [1R(1R*,3S*,5R*,6R*,9R*,11R*,15S*,16R*,17R*,18S*,19E,21E,23E,25E,27E,29E,31E,33R*,35S*,36R*,37S*)]-33-[(3-amino-3,6-dideoxy-ß-D-manno-pyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxa-bicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylic acid

STRUCTURAL FORMULA:



Molecular weight: 924.09
Empirical Formula: $C_{47}H_{73}NO_{17}$



(amphotericin B)

SUPPORTING DOCUMENTS:

BACKGROUND:

The subject of this NDA is AmBisome, a liposomal preparation of Amphotericin B, for the treatment and prophylaxis of systemic fungal infections, empiric therapy for presumed fungal infections and treatment of visceral leishmaniasis. AmBisome is an approved antifungal agent in the United Kingdom, some parts of Europe and Mexico.

Amphotericin B is a well known anti-fungal agent. An intravenous formulation of the drug is approved for the treatment of potentially life threatening fungal infections which include but are not limited to Aspergillosis, Cryptococcosis, Blastomycosis, systemic Candidiasis, Coccidioidomycosis, and Zygomycosis. Amphotericin B has an affinity for the sterol component of the cell membrane. Such a binding alters the membrane permeability leading to cell death.

SUMMARY:

The sponsor has investigated the activity of AmBisome *in vitro* and/or *in vivo* against (I) various fungal species, and (II) a protozoan parasite (*Leishmania*).

I. ANTI-FUNGAL ACTIVITY

Studies *in vitro*

The *in vitro* activity of AmBisome was tested by the macro- or micro-dilution method using RPMI 1640 medium against different fungal species which include *Candida*, *Cryptococcus*, *Aspergillus*

In the study by Anaissie, E. *et al.*, 1991 (Eur J Clin Microbiol Inf Dis 10: 665), 104 yeast isolates (*C. albicans*, *C. tropicalis*, *C. parapsilosis* and *Cr. neoformans*) and 21 mold isolates (*Aspergillus* sp. and *Fusarium* sp.) were tested according to NCCLS guidelines. Out of a total of 32 isolates of *C. albicans*, 11 were obtained from ATCC and 21 were isolated from the blood of cancer patients. The remaining isolates of other species, listed in Table 1, and most of the isolates listed in Table 2 were clinical isolates from patients with cancer. Different concentrations of the drug were incubated with the yeast form of different fungal species (10^4 cfu/ml) at 35 °C. The cultures were terminated after 48 hours for all species except *Candida* which were only incubated for a period of 24 hours. The lowest concentration of the drug which was effective in preventing visible growth of the organisms was termed as the minimum inhibitory concentration (MIC). The minimum fungicidal concentration (MFC) was determined by subculturing an aliquot (10 ul) from the wells showing no growth in primary culture. The results of the *in vitro* activity studies shown in Tables 1 and 2 indicate that MIC and MFC values for AmBisome are comparable to those observed using free amphotericin B.

Table 1: Activity of amphotericin B (AmB) and liposomal amphotericin B (lipo-AmB) against yeast isolates.

Fungal species (n)	Drug	MIC/MFC (µg/ml) MIC50	MIC90	Range	MFC50	MFC90	Range
Candida albicans (32)	AmB	1.25	1.25		1.25	1.25	
	lipo-AmB	0.62	0.62		0.62	1.25	
Candida tropicalis (20)	AmB	1.25	1.25		1.25	2.50	
	lipo-AmB	0.62	0.62		0.62	1.25	
Candida parapsilosis (20)	AmB	1.25	1.25		2.50	2.50	
	lipo-AmB	0.62	0.62		1.25	2.50	
Cryptococcus neoformans (32)	AmB	0.62	1.25		0.62	2.50	
	lipo-AmB	0.31	0.62		0.31	1.25	

Table 2: Activity of amphotericin B (AmB) and liposomal amphotericin B (lipo-AmB) against aspergilli and fusaria (MIC values did not differ among *Aspergillus* spp. or *Fusarium* spp.).

Fungal species (n)	Drug	MIC (µg/ml) MIC50	MIC90	Range
Aspergillus spp. (13)	AmB	2.50	2.50	
	lipo-AmB	1.25	1.25	
Fusarium spp. (8)	AmB	2.50	2.50	
	lipo-AmB	2.50	2.50	

In addition to the study described above, there were 8 reports of *in vitro* susceptibility testing of different fungal species using AmBisome and amphotericin B. The results of these studies, summarized in Table 3, show the *in vitro* activity of AmBisome and amphotericin B to be comparable despite the variations in activity from one lab to another.

The sponsor used an *in vitro* model of murine peritoneal macrophages infected with *Candida (Torulopsis) glabrata* to study the localization of labeled AmBisome as compared to empty liposomes. The results show that AmBisome containing amphotericin B at 25 and 40 ug inhibited the viability of yeast cells to 51% and 29% respectively. Viability was determined by staining with methylene blue. Of note, there was no comparison made to the free drug and traditional MIC values were not determined.

Table 3

In vitro Susceptibility of Fungi to AmBisome

Organism Tested	Amphotericin B Antimicrobial Activity		AmBisome Antimicrobial Activity		Reference
	MIC	MFC	MIC	MFC	
Tube Macrodilution Test Method					
C. albicans ATCC 44858	0.1 µg/mL (no visible growth)	3.2 µg/mL (>99 %)	0.05 µg/mL	6.4 µg/mL	[3]
Aspergillus fumigatus 4215	1.0 µg/mL ?	>4.0 µg/mL ?	1.0 µg/mL	>4.0 µg/mL	[4]
Blastomyces dermititidis ATCC 26199	0.5 µg/mL (no visible growth)	1.0 µg/mL (no growth on subculture)	0.125 µg/mL	1.0 µg/mL	[5]
Paracoccidioides brasiliensis, Gar	0.25 µg/mL (no visible growth)	0.5 µg/mL	1.0 µg/mL	2.0 µg/mL	[6]
Plate Microdilution Test Method					
C. albicans (32)	1.25 µg/mL[a]	1.25 µg/mL[b]	0.62 µg/mL[a]	1.25 µg/mL[b]	[7]
C. tropicalis (20)	1.25 µg/mL[a]	2.50 µg/mL[b]	0.62 µg/mL[a]	1.25 µg/mL[b]	
C. parapsilosis (20)	1.25 µg/mL[a]	2.50 µg/mL[b]	0.62 µg/mL[a]	1.25 µg/mL[b]	
C. neoformans (32)	1.25 µg/mL[a]	2.50 µg/mL[b]	0.62 µg/mL[a]	1.25 µg/mL[b]	
Aspergillus spp. (13)	2.50 µg/mL[a]		1.25 µg/mL[a]		
Fusarium spp. (8)	2.50 µg/mL[a]		2.50 µg/mL[a]		
C. albicans 620	0.62 µg/mL	0.62 µg/mL	0.62 µg/mL	0.62 µg/mL	[8]
C. krusei 2506	0.125 µg/mL[c]		0.125 µg/mL[c]		[9]
C. krusei 2506					[10]
C. krusei 4935	(				
C. lusitaniae 1706					
C. lusitaniae 524					
C. lusitaniae 5W31	1.25 µg/mL		1.25 µg/mL		
C. lusitaniae 2819	2.5 µg/mL		2.5 µg/mL		
Aspergillus fumigatus ATCC 13073	0.16 µg/mL ?		0.16 µg/mL ?		[11]

[a] MIC_{90}
[b] MFC_{90}
[c] MIC_{50}
[d] MIC_{range}

The time kinetics of drug activity for AmBisome and amphotericin B against *C. albicans* was compared in one study (van Etten *et al.*, 1993, J Antimicrobial Agents Chemother 32: 723) by measurement of *in vitro* susceptibility at different time points in culture. The results in Figure 1 show that the lower concentration of amphotericin B (0.2 ug/ml) kills the yeast form within 2 hours in culture. A > 10 fold higher concentration (25.6 ug/ml) of AmBisome and a longer incubation time (approximately 6 hours) were necessary to produce a similar fungicidal effect with this drug. However, at 24 hours the MFC was comparable for the two drugs (3.2 vs. 6.4 ug/ml).

Overall, these studies show that the *in vitro* activity of AmBisome against a wide range of fungal species is comparable to that of amphotericin B. Whether repeated exposure of the organisms to AmBisome *in vitro* will alter drug susceptibility is not known.

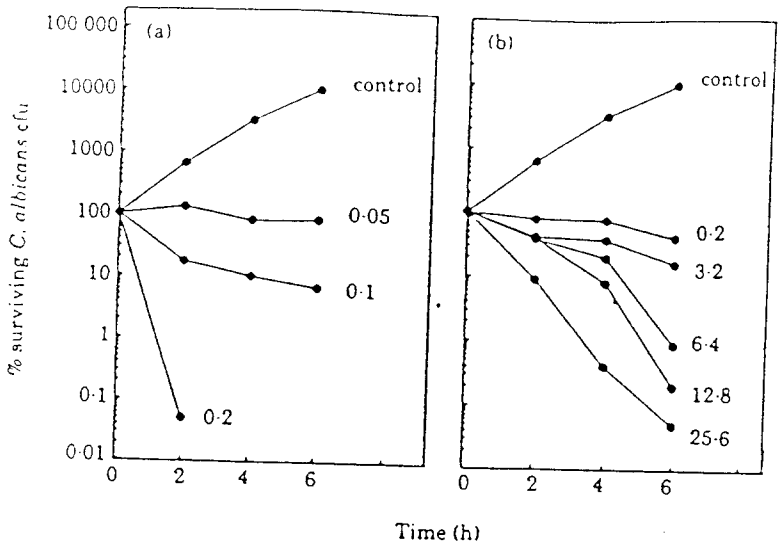


Figure 1. In-vitro fungicidal activities of AmB-DOC (a) and L-AmB (b) in varying concentrations (mg/L) against *C. albicans*. Each symbol represents the mean of three experiments.

Studies *in vivo*

A. *Candida albicans*

(a) Activity of AmBisome as a Prophylactic Agent against *C. albicans*

The activity of a single dose of AmBisome as a prophylactic agent was measured in both **immunocompetent** and **immunosuppressed** C57BL/6 mice infected with *C. albicans* (strain # 39; 10^6 and 10^5 yeast cells respectively) by the intravenous route (report no. 232-93108). Four days prior to drug administration, immunosuppression was initiated by administration of 75 mg/kg cyclophosphamide by the intraperitoneal (IP) route and continued every 3 days thereafter. A single dose of AmBisome, amphotericin B or the vehicle was administered 2, 4, or 7 days prior to infection. Mice were weighed prior to treatment and every 3 days thereafter. After 7 days of infection, the kidneys obtained from surviving mice were processed to measure fungal burden.

The studies in both **immunocompetent** (Table 4) and **immunosuppressed** (Table 5) mice show that a single dose of AmBisome was effective as a prophylactic agent in reducing the fungal burden in kidney. Such an effect was observed when the drug was administered 2, 4 or 7 days before infection. The activity of amphotericin B as a free drug vs. the liposomal preparation, at the same dose, was comparable. However, it is known that free amphotericin B is toxic when given at higher doses. These studies show that the liposomal preparation can deliver a higher total dose of amphotericin B to the reticuloendothelial system before toxicity is induced. The reduction in fungal burden increased in parallel with the increase in drug dose. Tissues other than kidney were not tested. Given that all the mice included in the study survived the 7 day period of observation, it would have been worthwhile to follow-up the mice beyond this time period to measure fungal burden and survival.

TABLE 4

Average Log CFU/g kidney (± SD)
(7 days post-infection) from Immunocompetent *Candida* infected C57BL/6 mice Prophylactically Treated with Fungizone or AmBisome

Treatment	-7 Day Prophylaxis	-4 Day Prophylaxis	-2 Day Prophylaxis
	Avg. Log CFU/g (± SD)	Avg. Log CFU/g (± SD)	Avg. Log CFU/g (± SD)
20 mg/kg AmBisome	3.3 (± 0.4)	3.1 (± 0.1)	2.6 (± 0.4)
10 mg/kg AmBisome	3.5 (± 0.3)	3.6 (± 0.4)	3.0 (± 0.5)
5 mg/kg AmBisome	3.8 (± 0.2)	4.0 (± 0.2)	3.8 (± 0.3)
1 mg/kg AmBisome	4.6 (± 0.5)	4.4 (± 0.2)	4.6 (± 0.3)
1 mg/kg Fungizone	5.2 (± 0.7)	5.4 (± 0.4)	4.9 (± 0.4)
Untreated	6.0 (± 0.4)	-----	-----

TABLE 5

Average Log CFU/g kidney (±SD)
(7 days post-infection) from immunosuppressed *Candida* infected C57BL/6 mice treated prophylactically with Fungizone or AmBisome

Treatment	-7 Day Prophylaxis	-2 Day Prophylaxis
	Avg. Log CFU/g (± SD)	Avg. Log CFU/g (± SD)
20 mg/kg AmBisome	2.7 (± 0.8)	2.4 (± 0.3)
5 mg/kg AmBisome	3.5 (± 0.7)	2.4 (± 0.4)
1 mg/kg AmBisome	3.2 (± 1.0)	4.6 (± 0.2)
1 mg/kg Fungizone	4.2 (± 1.2)	4.9 (± 1.2)
Untreated	6.2 (± 0.7)	-----

(b) Activity of AmBisome as a Therapeutic Agent against *C. albicans*

(i) Activity in immunocompetent mice

CD_2F_1 male mice were infected with *C. albicans* (3×10^5 cfu: strain 410) by the intravenous (IV) route (Gondal, J.A. *et al.*, 1989, Antimicrob Agents Chemother 33:1544). After 2 or 3 days of infection, the mice were treated with different doses of either amphotericin B or AmBisome by IV injection. The untreated mice died by 12 days post-infection with a median survival of 7 days. Results in Table 6 show the effects of treatment with a **single dose** of either the free drug or the liposomal preparation of amphotericin B on day 2 or 3 of infection. Administration of AmBisome

at a dose of ≥ 1 mg/kg on day 2 of infection significantly improved the percentage survival compared to the untreated control. However, when 1 mg/kg dose was administered on day 3 of infection (when one would expect the extent of infection to be more severe as compared to day 2), the median survival time decreased and was similar to the untreated control group (7 days). A reduction in activity was also observed at the higher dose of 2.5 or 5 mg/kg when treatment was initiated on day 3 as compared to day 2. For both dose groups 7/8 mice survived the 42 day period of observation when treatment was initiated on day 2. In contrast, when treatment was initiated on day 3, 3/8 animals in the 2.5 mg/kg group and 4/8 in the 5 mg group survived the 42 day period of observation with median survival of 26 and 42 days respectively. Higher dose levels (between 7.5 and 15 mg/kg) were effective in prolonging the survival of the majority of the animals irrespective of the time treatment was initiated (i.e., day 2 or 3). At the highest dose tested (i.e., 20 mg/kg), all 8 mice survived when treatment was initiated 2 days after infection. However, only one-half of the mice survived the 42 day period of observation when treatment was initiated 3 days post-infection. This observation could be due to toxicity. It would have been worthwhile if other time points for initiation of treatment (e.g., day 1, 4 and 5) had been included in the study to better determine how changes in the extent of infection impact the survival rate and the residual organism load.

The presence of residual organisms was measured in the kidneys of a limited number of mice from each group (described above) and the results are expressed as number of animals which tested positive (Table 6). Quantitative measurement of the number of colony forming units was not done. The authors have indicated that tissues other than kidney were processed for measurement of *Candida* organisms and no outgrowth was observed in liver, spleen and blood. The presence of organisms in brain was not tested. In untreated, infected mice, liver, spleen, blood and kidney tested positive for cfu.

TABLE 6. Effect of single doses of AMB or lip-AMB on survival of CD_2F_1 mice infected with *C. albicans*[a]

Group	Dose (mg/kg)	Day 2 treatment			Day 3 treatment		
		Median survival (days)	No. of survivors by day 42	*Candida* outgrowth in kidney (no. positive/no. tested)	Median survival (days)	No. of survivors by day 42	*Candida* outgrowth in kidney (no. positive/no. tested)
Control		7.0	0		7.0	0	ND[b]
AMB	0.5	23.5	3	2/3	11.5	0	ND
	1.0	18.0	1	0/1	18.0	0	ND
	1.5	18.0	1	0/1	8.5	0	ND
	2.0	21.5	2	0/2	ND	ND	ND
Lip-AMB	1.0	34.5	3	3/3	7.0	0	
	2.5	—[c]	7*†	2/3	26.0	3	3/3
	5.0	—	7*†	1/3	42.0	4*†	3/3
	7.5	—	7*†	0/3	—	6*†	0/3
	10.0	—	8*†	1/3	—	8*†	3/3
	15.0	—	6*†	0/3	—	8*†	2/3
	20.0	—	8*†	0/3	42.0	4*†	1/3

[a] CD_2F_1 mice (eight mice per group) were injected with 3.5×10^5 *C. albicans*, i.v., via the tail vein. On day 2 or 3 postinfection, mice were treated i.v. via the tail vein with either HBSS (control), AMB, or lip-AMB. Symbols: *, $P < 0.02$ versus control; †, $P < 0.02$ versus AMB at 0.5 mg/kg.

[b] ND, Not determined.

[c] —, >42 days.

In another experiment (Gondal, J.A. *et al.*, 1989, Antimicrob Agents Chemother 33:1544), mice were administered **multiple doses** of the drugs on days 3, 6 and 9 of infection. Results in Table 7 indicate that the majority of the mice treated with 3 doses of ≥ 5 mg/kg survived the 42 day period of observation. Mice treated with the free drug had a median survival time of 30-33 days. Residual organisms were detected in some of the mice treated with either the free drug or the liposomal preparation of amphotericin B. However, the number of mice studied were far too small to support any definitive conclusions. Here again, the actual colony count was not provided. The results of residual organism counts in organs other than kidneys also were not provided, but it was stated that no viable colonies were detected.

TABLE 7 Effect of multiple doses of AMB or lip-AMB on survival of CD_2F_1 mice infected with *C. albicans*[a]

Group	Dose (mg/kg)	Median survival (days)	No. of survivors at day 42/no. in group	*Candida* outgrowth in kidney (no. positive/no. tested)
Control		7.0	0/8	
AMB	1.0	33.0	3/8	2/3
	1.5	30.0	2/8	3/3
Lip-AMB	5.0	—[b]	12/13*†‡	2/3
	6.5	—	12/13*†‡	2/3
	8.5	—	13/13*†‡	1/3

[a] CD_2F_1 mice were injected with 3.5×10^5 *C. albicans*, i.v., via the tail vein. At 3, 6, and 9 days postinfection, mice were treated i.v. via the tail vein with either HBSS (control), AMB, or lip-AMB. Symbols: *, $P < 0.02$ versus control; †, $P < 0.02$ versus AMB at 1.0 mg/kg; ‡, $P < 0.01$ versus AMB at 1.5 mg/kg.

[b] —, >42 days.

In another study by Adler-Moore, J.P. *et al.* (J Antimicrob Chemother 1991, 28 [Suppl B]: 63 and study report no. 232-89106 and 232-89109), C57BL/6 female mice were infected by intravenous inoculation of 3 to 4 x 10^6 organisms of *C. albicans*. After 3 days of infection, mice were administered either the free drug or the liposomal preparation of amphotericin B for 1 to 5 days. The animals were observed for survival rate for a period of 21 to 41 days. The presence of residual fungal load was determined in the kidneys on day 22 of infection. Treatment with a **single dose** of AmBisome showed a decrease in the organism load in kidneys at all doses tested as compared to the untreated control group (Table 8). An improvement in the percentage survival after treatment with AmBisome was also observed compared to the untreated animals. Treatment with the free drug improved the percentage survival but the organism load in the kidney was not significantly altered. As observed in the single dose study, treatment with **multiple doses** of AmBisome improved the survival and / or reduced the organism load (Table 9). However, it should be noted that a different placebo was used in the 2 experiments (phosphate buffer saline vs. 9% sucrose in succinate buffer) and the mean number of CFU counted in the control group in this study was about 5 to 6 fold less than that observed in the single dose study (compare Tables 8 and 9). No reason for the observed difference in organism load between the 2 experiments was provided. Other organs were not tested.

In another experiment, mice were infected and treated as described above, however, survival was followed for a period of 41 days. AmBisome (VS104) was effective in improving the survival of these infected mice (Table 10). The fungal burden was not measured in this study.

Table 8. Kidney clearance and survival of *C. albicans*-infected mice following single treatment with conventional amphotericin B or AmBisome

Treatment[a]	cfu/mg kidney (range)	Survivors/inoculated
Phosphate buffered saline (control)	28,300	4/8
Fungizone (0.75 mg/kg)	22,750	7/8
AmBisome (2.5 mg/kg)	200	8/8
AmBisome (5.0 mg/kg)	50	8/8
AmBisome (10.0 mg/kg)	7	8/8

[a]Intravenous treatment three days after challenge; [b]$n = 6$; [c]$n = 8$.

Table 9. Kidney clearance and survival of *C. albicans*-infected mice following multiple dose treatment with conventional amphotericin B or AmBisome

Treatment[a]	cfu/mg kidney (range)	Survivors/Inoculated
9% sucrose, 10 mM sodium succinate, pH 5.5	5074	3/5
Fungizone (0.75 mg/kg)	1019	4/5
AmBisome (2.5 mg/kg)	68	5/5
AmBisome (5.0 mg/kg)	16	5/5
AmBisome (10 mg/kg)	9	5/5

[a]Intravenous treatment given for five consecutive days beginning three days after challenge; survivors killed 22 days after infection; $n = 5$.

TABLE 10

Survival of C57BL/6 Mice Following Intravenous Challenge with *Candida Albicans* and Multidose Treatment with VS104 (AmBisome)

Treatment[a]	Day of Death Post-Infection	Long-Term Survivors
	Subgroup A	41 Days Post-Infection
Control (Sucrose solution buffered with sodium succinate)	10, 20, 28, 34, 34	0/5
Conventional amphotericin B (0.75 mg/kg)	6, 10, 34	2/5
VS104 (2.5 mg/kg)	>41	5/5
VS104 (5.0 mg/kg)	17	4/5
VS104 (10.0 mg/kg)	>41	5/5
[a]Mice were given indicated treatments IV for five consecutive days beginning three days post-infection.		

In another study by the same group of workers (Adler-Moore, J.P. *et al.* 1991, J Antimicrob Chemother, 28 [Suppl B]: 63), treatment with AmBisome was initiated on day 2 post-infection for a period of 5 days. One week after discontinuation of treatment (i.e., 2 weeks post-infection), the animals were sacrificed to determine the organism load in the kidney. Results shown in Table 11 indicate a significant reduction in the cfu/mg kidney after treatment with the liposomal preparation of the drug. Treatment with the free drug also reduced the organism load similar to that observed using the liposomal formulation. It should be noted that in this experiment, animals were sacrificed about a week after treatment was discontinued. This is in contrast to the previous study (see Table 9) in which animals were sacrificed 2 weeks after treatment. Also, the drug concentrations were measured 24 hours after discontinuation of treatment and not at the time residual fungal burden was measured.

Table 11 . Kidney and serum concentrations of amphotericin B, and cfu of candida in the kidneys of *C. albicans*-infected mice following treatment with Fungizone or AmBisome

	Amphotericin B				
	kidney (μg/g)		Plasma (mg/L)		*C. albicans*
Treatment[a]	24 h	8 day	24 h	8 day	cfu/mg kidneys (range) (14 day)
Control	...	...	...	...	86,000
Fungizone (0·75 mg/kg	3·7	1·1	0·24	0·00	92
AmBisome (0·75 mg/kg)	1·3	0·6	0·19	0·07	122
AmBisome (5·0 mg/kg)	5·4	2·6	0·25	0·16	60

[a]Intravenous treatment given for five consecutive days beginning two days post-challenge; mice were killed 14 days post-infection; $n = 5$.

Studies by van Etten *et al.*, 1993 (J Antimicrob Chemother 32: 723), were conducted in Balb/c mice infected with *C. albicans* (5 x 10^4 cfu: ATCC strain 44858) by the IV route. Daily treatment with IV AmBisome or amphotericin B (at maximum tolerated dose) was initiated 48 hours after infection and continued for 5 days. Fluconazole was administered 2 times daily by intraperitoneal route. Animals were followed for survival for up to 14 days after discontinuation of treatment and fungal burden was measured in the kidneys immediately after the start of treatment, and at 1 and 14 days after discontinuation of treatment. The kidneys were also examined for the presence of hyphal growth by microscopic examination of periodic acid-Schiff stained tissue sections. These investigators focused their evaluation on fungal load in the kidney, based on previous observations that show the infection is confined to the kidneys in immunocompetent mice. Results in Table 12 show that AmBisome was effective in reducing fungal burden. It appears that the majority of mice used in the study survived the period of observation. The reduction in cfu in mice treated with AmBisome at a 0.4 mg/kg dose was comparable to the amphotericin B treated group at the same dose which was shown to be equivalent to the maximum tolerated dose of the free drug in this model. The results also show a decrease in fungal burden 14 days after discontinuation of treatment with 7 mg/kg AmBisome. However, it is possible that high concentrations of the drug may still be present in the tissue as was shown in the immunosuppressed mouse model (see below). Drug concentrations were not measured in immunocompetent mice.

Table 12. Effects of treatment on growth of *C. albicans* in the kidneys of immunocompetent mice

Treatment[a]	Dosage (mg/kg/day)	Start of treatment $\log_{10}$ cfu/kidney	24 h after termination of treatment: hyphal growth	24 h after termination of treatment: $\log_{10}$ cfu/kidney in surviving mice	14 days after termination of treatment: hyphal growth	14 days after termination of treatment: $\log_{10}$ cfu/kidney in surviving mice	14 days after termination of treatment: no. of mice with sterile kidneys/no. of surviving mice
		4·58 ± 0·29					
AmB-DOC	0·4		—[c]	2·44 ± 0·32[e]	—	2·15 ± 0·88	0/10
L-AmB	0·4		—	3·06 ± 0·62[e]	—	2·81 ± 1·18	0/10
L-AmB	7		—	2·20 ± 0·87[e]	—	0·40 ± 0·25	1/10
Fluconazole	0·4		—	3·57 ± 1·10[e]	+	4·90 ± 1·32[f]	0/10
Fluconazole	64		—	2·97 ± 0·58[e]	+	4·41 ± 1·39[f]	0/10
Placebo-DOC			+[d]	4·19 ± 0·62	+	6·08 ± 0·85[f]	0/10
Placebo-Liposomes			+	4·78 ± 0·79	+	6·67 ± 0·80[f]	0/10
Placebo-PBS			+	4·40 ± 0·76	+	6·56 ± 0·65[f]	0/10

[a]Started 48 h after inoculation and continued for 5 days.
[b]Geometric mean ± S.D.
[c]No hyphal growth; [d] Hyphal growth.
[e]$P \leq 0.025$ compared with number of cfu at start of treatment.
[f]$P \leq 0.025$ compared with number of cfu 24 h after termination of treatment.

(ii) Activity in immunosuppressed mice

Balb/c mice were immunosuppressed with cyclophosphamide (100 mg/kg was administered 4 days prior to infection and 75 mg/kg at 3 day intervals thereafter) and infected with *C. albicans* (10^4 cfu: ATCC strain 44858) by the IV route (van Etten *et al.*, 1993, J Antimicrob Chemother 32: 723). The authors stated that such a regimen resulted in granulocytopenia (<0.1 x 10^9 cells/liter) at or before the infection. Treatment with AmBisome, amphotericin B (by the IV route) or fluconazole (by the IP route) was initiated 6, 16 or 24 hours after infection for 5 days. Fungal burden was measured in kidneys, liver, spleen and lungs. Unlike immunocompetent mice (page 10), the majority of the untreated leukopenic mice infected with *C. albicans* died by 19 days of infection (Table 13). Treatment with AmBisome (7 mg/kg) was effective in improving the survival and reducing the fungal burden in kidney (Tables 14 and 15) and other tissues (data not shown). The reduction in cfu was observed 14 days after discontinuation of treatment. However, at this time high concentrations of the drug were measurable in the tissues (Table 16). The fungal burden after clearance of residual drug from the tissues was not measured.

Table 13 Effects of treatment on survival of leucopenic mice infected with *C. albicans*

Treatment[a]	Dosage (mg/kg/day)	Per cent survivors: 24 h after termination of treatment	Per cent survivors: 14 days after termination of treatment
AmB-DOC	0·3	100	100
L-AmB	0·3	90	50
L-AmB	7	100	100
Fluconazole	0·3	60	0
Fluconazole	64	100	100
Placebo-DOC		80	0
Placebo-Liposomes		80	10
Placebo-PBS		40	0

[a]Started 6 h after inoculation and continued for 5 days.

Table 14. Effects of treatment on growth of *C. albicans* in the kidneys of leucopenic mice

Treatment[a]	Dosage (mg/kg/day)	Start of treatment $\log_{10}$ cfu/kidney	24 h after termination of treatment: hyphal growth	24 h after termination of treatment: $\log_{10}$ cfu/kidney in surviving mice	14 days after termination of treatment: hyphal growth	14 days after termination of treatment: $\log_{10}$ cfu/kidney in surviving mice	14 days after termination of treatment: no. of mice with sterile kidneys/no. of surviving mice
		2·65 ± 0·22					
AmB-DOC	0·3		—	2·81 ± 0·49	+	5·28 ± 1·14[d]	0/10
L-AmB	0·3		...[b]	4·20 ± 0·67	...	3·96 ± 1·86	0/5
L-AmB	7		—	1·21 ± 0·30[c]	—	0·39 ± 0·52	6/10
Fluconazole	0·3		...	5·83 ± 0·61	...	...	...
Fluconazole	64		—	1·99 ± 0·47[c]	+	5·20 ± 1·28[d]	0/10
Placebo-DOC			...	5·67 ± 1·10	...	...	...
Placebo-Liposomes			...	5·60 ± 0·93	...	5·56	0/1
Placebo-PBS			...	6·70 ± 0·48	...	...	...

[a]Started 6 h after inoculation and continued for 5 days.
[b]Not investigated.
[c]$P \leq 0·025$ compared with number of cfu at start of treatment.
[d]$P \leq 0·025$ compared with number of cfu 24 h after termination of treatment.

Table 15 Effects of treatment in relation to severity of infection, assessed in terms of survival and growth of *C. albicans* in the kidneys of leucopenic mice

Start of treatment (after inoculation)	Treatment[a]	Dosage (mg/kg/day)	Per cent survivors[b]	hyphal growth[b]	Log_{10} cfu/kidney in surviving mice[b]	No. of mice with sterile kidneys/no. of surviving mice[b]
6 h	AmB-DOC	0·3	100	+	5·28 ± 1·14	0/10
	L-AmB	7	100	—	0·39 ± 0·52	6/10
	fluconazole	64	100	+	5·20 ± 1·28	0/10
16 h	AmB-DOC	0·1	40	...	6·20 ± 0·97	0/4
	L-AmB	7	100	—	1·90 ± 0·66	0/10
	fluconazole	64	70	...	6·17 ± 0·61	0/7
24 h	AmB-DOC	0·1	...[c]	...	...	...
	L-AmB	7	90	...	1·29 ± 0·82	0/9
	fluconazole	64	...	...	...	...

[a]For 5 days.
[b]Determined 14 days after termination of treatment.
[c]Not investigated.

Table 16 AmB concentrations in blood and tissues after treatment of infected leucopenic mice with AmB-DOC or L-AmB

Treatment[a]	Dosage (mg/kg/day)	AmB concentration[b] 24 h after termination of treatment: blood mg/L	liver mg/kg	spleen mg/kg	kidney mg/kg	lung mg/kg	AmB concentration[b] 14 days after termination of treatment: blood mg/L	liver mg/kg	spleen mg/kg	kidney mg/kg	lung mg/kg
AmB-DOC	0·3	0·2	ND[c]	3·3	ND	0·6	ND	ND	2·3	ND	ND
L-AmB	7	1·7	356	700	14·5	5·9	ND	170	243	7·8	ND

[a]Started 6 h after inoculation and continued for 5 days.
[b]Blood and tissue samples from five mice were pooled.
[c]Not detectable.

The survival data and fungal burden for groups wherein treatment was initiated at 16 or 24 hours post-infection was similar (Table 15). The cfu count in the group wherein treatment was initiated at 24 hours post-infection was about 1 log higher than that observed in the group of mice in which treatment was initiated at 6 hours post-infection. It would have been worthwhile to measure the activity of the drug in a more severe infection by initiating the treatment 48 or 72 hours post-infection or by increasing the concentration of the initial inoculum. The investigators have stated that delaying the time of treatment from 6 to 16 or 24 hours was effective in reducing the MTD dose thereby indicating an increase in the toxicity of the drug in mice with severe infection.

In another experiment, the effect of initial treatment with AmBisome (initiated 6 hours after infection) for 5 days followed by prolonged treatment with fluconazole for 13 days was examined. Results in Table 17 show that maintenance therapy with AmBisome + fluconazole did not alter the outcome as compared to the group treated with AmBisome alone.

Table 17. Effects of prolonged or maintenance treatment with fluconazole on growth of *C. albicans* in the kidneys of leucopenic mice

Treatment[a]	Dosage (mg/kg/day)	Log_{10} cfu/kidney in surviving mice[b]	No. of mice with sterile kidneys/no. of surviving mice[b]
Fluconazole[c]	64	2·28 ± 0·36	0/10
L-AmB[d]	7	0·39 ± 0·52	6/10
L-AmB/Fluoconazole[e]	7/64	0·25 ± 0·41	7/10

[a]Started 6 h after inoculation.
[b]Effects of treatment were assessed 19 days after starting.
[c]Administered for 18 days.
[d]Administered for 5 days.
[e]L-AmB for 5 days followed by fluconazole for 13 days.

B. Other *Candida* species

The activity of AmBisome was measured against other species of *Candida* (experimental hematogenous infections) which include *C. krusei* (2 strains) and *C. lusitaniae* (4 strains - 2 of which were known to be resistant to amphotericin B) in immunosuppressed CF1 mice (Karyotakis and Anaissie, 1994 Antimicrob Agents Chemother 38: 2660). The authors have stated that the experimental design was similar to that used in another study by Anaissie *et al.*, 1994 (J Inf Dis 170: 384). In this cross-referenced study the authors stated that the concentration of the inoculum used for infection by the IV route was standardized so that the median survival time of infected mice was between 5 and 9 days. The mice were immunosuppressed 24 hours prior to infection. For *C. lusitaniae* infection, mice were immunosuppressed with cortisone acetate (for 3 days) and 5-fluorouracil (single dose); whereas those infected with *C. krusei* were administered a single dose of cortisone acetate + cyclophosphamide. The leukocyte count in the immunosuppressed mice was not shown. Treatment with IV AmBisome, amphotericin B or the vehicle was initiated 1 hour after infection and continued for 3 days. The fungal burden was measured in the kidneys 24 hours after discontinuation of treatment in a few mice. The remaining mice were followed for survival for a period of 21 days post-infection. Results in Table 18 show that the strains of *C. krusei* and *C. lusitaniae* susceptible to amphotericin B were also susceptible to AmBisome. The highest dose of AmBisome (30 mg/kg) used in the experiment, however, appeared to be toxic. It is of note that the immunosuppression caused by cortisone acetate, 5-fluorouracil or cyclophosphamide is transient and is easily reversed by discontinuation of these immunosuppressive agents. Therefore, the activity of the drug as measured by survival rate (on day 21) or fungal burden (on day 4) may also be due to the recovery of leukocyte numbers.

TABLE 18 Activities of AmB and LiAmB in neutropenic CF1 mice infected with two *C. krusei* and four *C. lusitaniae isolates*

Therapy[a]	*C. krusei 2506*		*C. krusei 4935*		*C. lusitaniae 1706* (S)		*C. lusitaniae 524* (S)		*C. lusitaniae 2819* (R)		*C. lusitaniae SW31* (R)	
	Median survival (days [%[b]])	Mean CFU/g[c]	Median survival (days [%])	Mean CFU/g	Median survival (days [%])	Mean CFU/g	Median survival (days [%])	Mean CFU/g	Median survival (days [%])	Mean CFU/g	Median survival (days [%])	Mean CFU/g
None	9 (32)	6.65	7 (20)	6.10	7 (23)	6.71	7 (12)	7.38	8 (23)	6.44	7 (23)	6.90
AmB												
2 mg/kg i.p.	21 (87)[d]	4.99[d]	21 (100)[d]	5.35[d]	NT[e]	NT	NT	NT	NT	NT	NT	NT
1 mg/kg i.v.	NT	NT	NT	NT	21 (78)[d]	3.73[d]	21 (95)[d]	3.21[d]	8 (23)	5.54	8 (23)	6.04
LiAmB												
8 mg/kg i.v.	21 (72)[d]	4.67[d]	17 (80)[d]	4.54[d]	NT	NT	NT	NT	NT	NT	NT	NT
10 mg/kg i.v.	NT	NT	NT	NT	21 (100)[d]	3.81[d]	21 (92)[d]	3.07[d]	10 (40)	5.42[d]	8 (0)	6.76
15 mg/kg i.v.	21 (70)[d]	3.70[d,f]	21 (75)[d]	4.07[d,f]	NT	NT	21 (80)[d]	1.00[d,f]	NT	NT	NT	NT
30 mg/kg i.v.	7 (27)	3.62[d,f]	NT	NT	21 (62)[d]	1.42[d,f]	NT	NT	10 (23)	5.33[d]	7 (20)	5.81[d]

[a] i.p. intraperitoneally; i.v., intravenously.
[b] Percent overall survival.
[c] Mean $\log_{10}$ CFU per gram of kidney tissue.
[d] $P < 0.05$ versus no therapy (Mann-Whitney U test).
[e] NT, not tested.
[f] $P < 0.05$ versus AmB therapy (Mann-Whitney U test).

In another study by Karyotakis et al., 1993 (J Inf Dis 168: 1311), CF1 mice were immunosuppressed and infected with *C. krusei* (3 x 10^7 cfu) as described above. Treatment with AmBisome, amphotericin B or the vehicle was initiated an hour after infection by the IV route for 3 days. Mice were followed for a period of 21 days for survival. Fungal burden in the kidney was measured on day 4 i.e., a day after discontinuation of treatment. Results in Figures 2 and 3 show that Amphotericin B and AmBisome (up to a dose of 15 mg/kg) were effective in improving the survival and reducing the fungal burden of infected mice. The highest dose tested (30 mg/kg) was effective in reducing the fungal burden, but was otherwise shown to be toxic. Here again, the role of leukocytes in the recovery from infection cannot be ruled out.

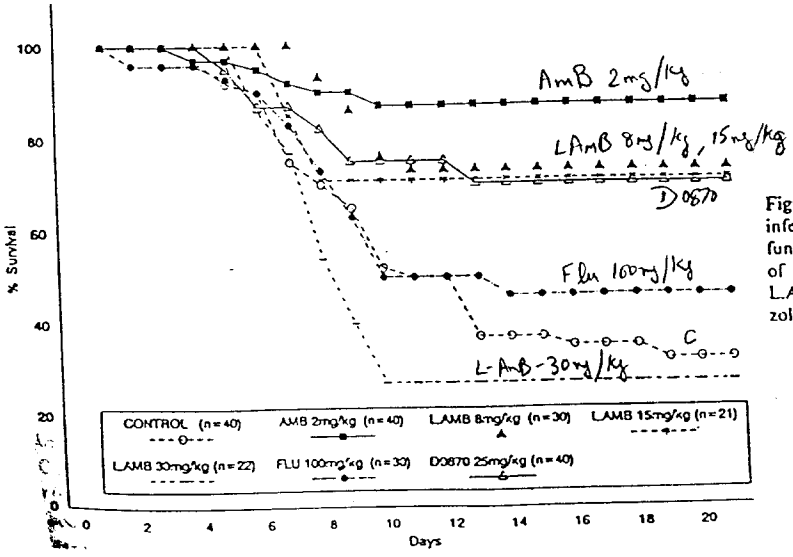


Figure 2 Survival curves of *C. krusei*-infected mice who received different antifungal regimens or no therapy. Day 1, day of challenge. AMB: amphotericin B. L.AMB: liposomal AMB. FLU: fluconazole.

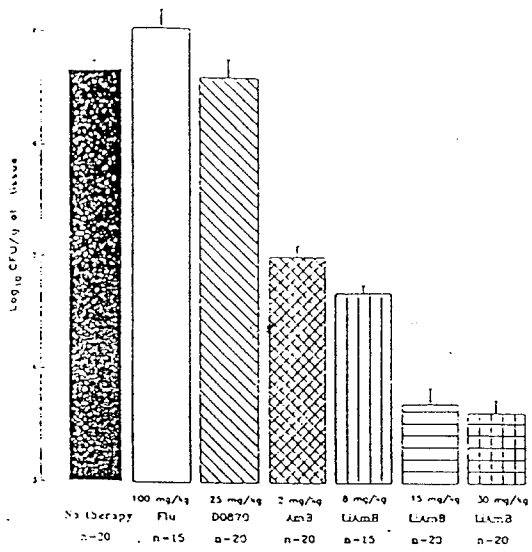


Figure 3. Mean cfu/g of kidney tissue (log_{10} scale) for mice infected with *C. krusei*. AMB, amphotericin B; LiAMB, liposomal AMB; Flu, fluconazole.

C. *Aspergillus*

(a) Activity of AmBisome as a Prophylactic Agent against *A. fumigatus*

ICR mice were immunosuppressed with cortisone acetate (125 mg/kg by subcutaneous route) for 3 days and 24 hours later infected with *A. fumigatus* (at different concentrations) by the intranasal route (Allen *et al.*, 1994, J Antimicrob Chemother 34: 100). AmBisome, amphotericin B or 5% dextrose were nebulized and administered 3, 2 and 1 days prior to infection. Each dose was administered over a period of 1 hour. Animals were observed over a period of 9 days for survival. Mice were then sacrificed and tissues processed for fungal culture. Results in Figure 4 show that AmBisome or amphotericin B, when administered as prophylactic agents were effective in improving the survival of infected animals. Such differences were statistically significant in groups of mice administered 10^8 or 10^7 organisms. AmBisome was also effective in reducing the fungal burden in the lung (Table 19).

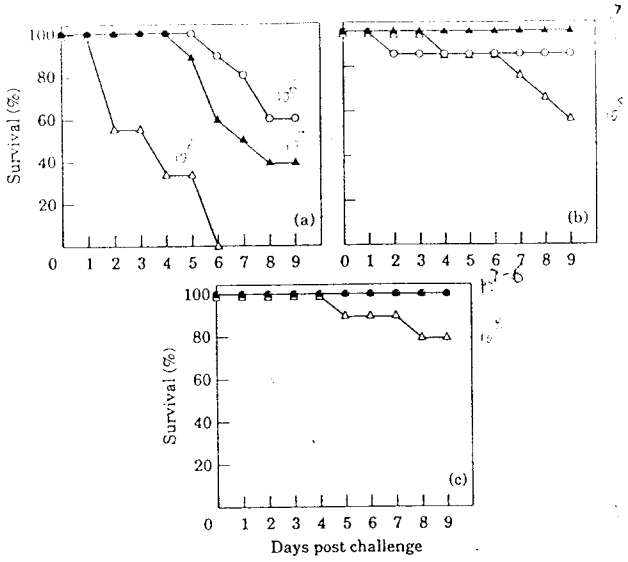


Figure 4. Survival over 9 days for animals challenged and treated with (a) 5% dextrose, (b) AmBisome, and (c) fungizone. Challenge 10^8 (△), 10^7 (▲), and 10^6 (○).

Table 1 Quantitative lung cultures taken on day 9 post-challenge

Treatment	Mean cfu (log_{10}/g of tissue (S.D.)) challenge 10^6	challenge 10^7	challenge 10^8
Challenge controls (D_5W)	4·67 (0·32)	5·30 (0·26)	N.S.[d]
AmBisome	0[a]	0·54 (1·20)[b,c]	4·62 (0·64)
Fungizone	0[a]	3·31 (0·77)[b]	4·59 (2·57)

[a] $P < 0.0005$, compared with challenge controls.
[b] $P < 0.05$, compared with challenge controls.
[c] $P < 0.05$, compared with Fungizone.
[d] NS, No survivors at day 9.

(b) Activity of AmBisome as a Therapeutic Agent against *A. fumigatus*

The activity of AmBisome against *A. fumigatus* as a therapeutic agent was measured in immunosuppressed rabbits (Francis *et al.*, 1994, J Inf Dis 169: 356). Rabbits were immunosuppressed with cytosine arabinoside (525 mg/m^2 by the IV route on days 1-5, 8 and 9) to produce persistent granulocytopenia and methyl prednisolone (5 mg/kg on days 1 - 3) to inhibit macrophage phagocytic function. Granulocyte count was < 500/ul from days 1 - 4 and < 100/ul from day 5 onward. The animals were also administered antibiotics to prevent bacterial infection. Rabbits were infected by the intratracheal route with conidia of *A. fumigatus* (1.5 - 2 x 10^8; the concentration was based on some pilot experiments showing this concentration to be optimal in terms of producing mortality, lesions, and culture positivity) on day 2 of cytosine arabinoside administration. From a practical standpoint the viscosity of the inoculum was appropriate for administering intratracheally. Treatment with AmBisome or amphotericin B was initiated 24 hours after infection for 10 days by the IV route and the survival measured. The untreated group was used as a control. Surviving animals were then sacrificed 24 hours after discontinuation of therapy. Lungs were examined for lesions and also for the presence of fungal burden. Bronchoalveolar lavage or serum samples were examined for host and cellular elements and analyzed for fungal metabolites which include mannitol, ergosterol and galactomannan. Pilot studies show that dissemination of infection to extra pulmonary sites occurred but was not consistent in terms of the organ involved (the tissues examined were liver, spleen, kidney and brain). Results in Figure 5 show the untreated rabbits died by 10 days of infection. However, majority of the rabbits treated with 1, 5 or 10 mg/kg of AmBisome survived the 10 day observation period. Amphotericin B at 1 mg/kg was less effective. AmBisome was also shown to be effective in reducing the fungal burden in lungs, pulmonary lesions and organ weight (Figure 6).

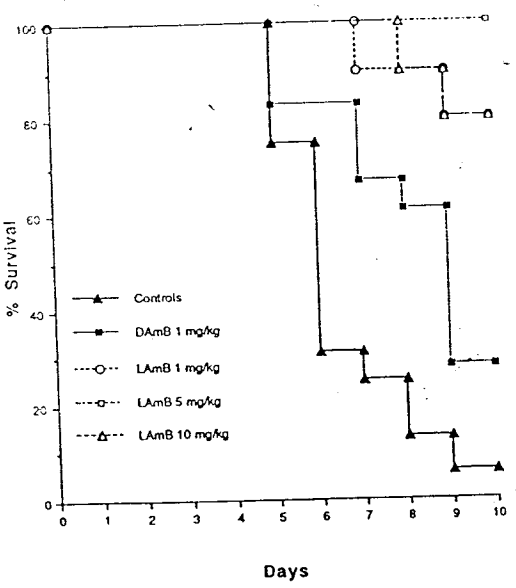


Figure 5 Kaplan-Meier plot of treatment groups of persistently granulocytopenic rabbits with primary pulmonary aspergillosis. Survival at 10 days of antifungal therapy was significantly improved for liposomal amphotericin B (LAmB) 10 mg/kg/day (n = 12), 5 mg/kg/day (n = 5), and 1 mg/kg/day (n = 10) compared with desoxycholate amphotericin B (DAmB) 1 mg/kg/day (n = 18) (P < .01) and untreated controls (P < .0001).

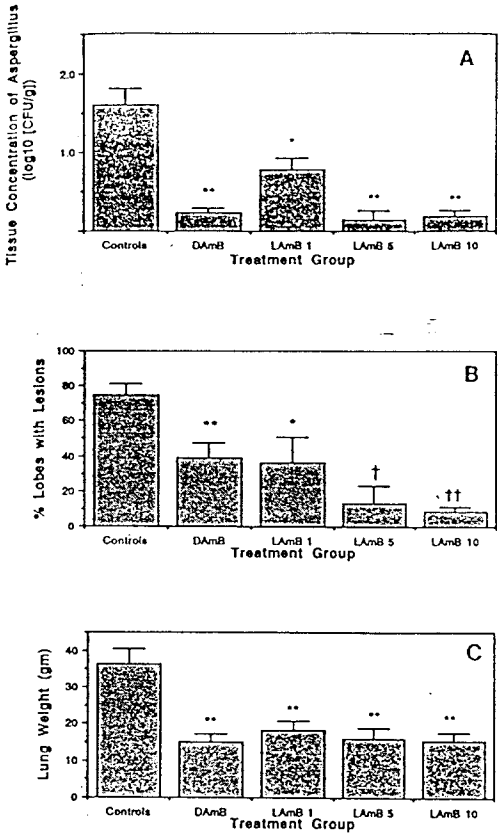


Figure 6 Response of primary pulmonary aspergillosis in rabbits to antifungal therapy measured by pulmonary tissue concentration of organisms (A), pulmonary hemorrhage score (B), and mean lung weights (C) in untreated controls (n = 18) and in groups treated with desoxycholate amphotericin B (DAmB) at 1 mg/kg/day (n = 18) or liposomal amphotericin B (LAmB) at 1 mg/kg/day (n = 10), 5 mg/kg/day (n = 5), or 10 mg/kg/day (n = 12). Compared with untreated controls (A–C): *, P < .05; **, P < .01; †, P < .001; ††, P < .0001.

Of the metabolites measured, mannitol (in bronchoalveolar lavage) and galactomannan (in serum) showed higher concentrations in infected animals compared to uninfected controls. These levels were reduced by treatment with amphotericin B or AmBisome (Figure 7 and Table 20). However, the usefulness of these markers in measuring drug activity is not established, consequently, these results should be interpreted with caution.

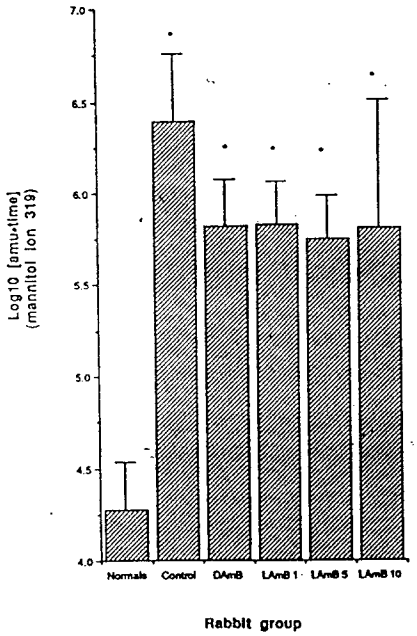


Figure 7 Detection of D-mannitol ion of 319 amu in bronchoalveolar lavage fluid of normal noninfected rabbits (n = 3), infected and untreated control rabbits (n = 5), and infected rabbits treated with desoxycholate amphotericin B, 1 mg/kg/day (DAmB; n = 6) or liposomal amphotericin B (LAmB) 1 mg/kg/day (n = 4), 5 mg/kg/day (n = 4), or 10 mg/kg/day (n = 5). *, P < .01 compared with normal noninfected controls. Antifungal therapy tended to reduce amount of D-mannitol in lavage fluid.

Table 20 Galactomannan antigenemia in persistently granulocytopenic rabbits with primary pulmonary aspergillosis.

	Untreated controls	Treated with LAMB or DAmB
Rabbits with detectable antigenemia/total (%)	10/14 (71)*	2/19 (10)
Plasma samples with detectable antigenemia/total (%)	12/45 (27)	6/24 (25)
Median onset of antigenemia after inoculation (days)†	6	6
Titer range‡		

NOTE. LAMB, liposomal amphotericin B: 1, 5, and 10 mg/kg/day; DAmB, desoxycholate amphotericin B: 1 mg/kg/day.
* $P \leq .001$.
† Among antigen-positive animals.
‡ Determined by serial 1:2 dilutions of latex agglutination EIA.

D. *Cryptococcus neoformans*

Female immunocompetent mice (NIH general purpose mice) were infected by intravenous inoculation of 6.5 x 10^6 organisms of *Cryptococcus neoformans* (Adler-Moore, J.P. *et al.*, 1991, J Antimicrob Chemother 28 [Suppl B]: 63; study report no. 232-89107). Starting three days post-infection, the mice were treated 3 times a week for 4 weeks with different doses of either the free drug (administered IP) or the liposomal formulation of amphotericin B (administered IV). The untreated control mice died within 8 to 16 days of infection with a mean survival of 12.1 days. It has been stated that the majority of the mice treated with either amphotericin B or AmBisome survived the 32 day period of observation (only 1 mouse in the group treated with 10 mg/kg of AmBisome died on day 20 of infection). The raw data were not provided. Residual organisms were not detected in the liver or spleen of AmBisome treated mice. However, residual organisms were observed in the spleen of mice treated with amphotericin B and also in the brain of some of the AmBisome and amphotericin B treated mice (Table 21). Here again, the colony count was not provided.

Table 21 Therapeutic efficacy of amphotericin B and AmBisome against murine cryptococcosis

Drug treatment	Dose* (mg/kg/dose)	Organs with positive cultures/total animals brain	liver	spleen	any organ
Fungizone	1·5	5/10	2/10	4/10	8/10
Fungizone	4·5	2/10	0/10	2/10	4/10
AmBisome	5·0	3/10	0/10	0/10	3/10
AmBisome	7·5	3/10	0/10	0/10	3/10
AmBisome	10·0	1/9	0/9	0/9	1/9

*Three doses per week for four weeks; Fungizone given intraperitoneally, AmBisome given intravenously.

The concentrations of amphotericin B in plasma and tissues (spleen, liver, kidney, lung and brain) were determined in uninfected animals receiving the highest dose of AmBisome (10 mg/kg) 30 minutes and 48 hours after 11 or 12 injections of the drug. Results in Table 22 show high concentrations of the drug in spleen and liver at both of the time points tested. Although high concentrations of the drug were measured in plasma, kidneys and brain 30 minutes after discontinuation of treatment, the levels were decreased by 48 hours particularly in plasma and brain. Drug concentrations in infected mice were not measured.

Table 22
Plasma Levels and Tissue Concentrations of Amphotericin B following AmBisome Treatment[a]

	0.5 Hours Post Injection	48 Hours Post Injection
Plasma	45.0 µg/mL	0.24 µg/mL
Liver	33.0 µg/gm	33.0 µg/gm
Spleen	36.5 µg/gm	30.5 µg/gm
Lungs	13.7 µg/gm	5.6 µg/gm
Kidneys	13.1 µg/gm	8.5 µg/gm
Brain	0.9 µg/gm	0.3 µg/gm

[a]Eight uninfected mice were given 10 mg/kg AmBisome intravenously three times per week for 4 weeks. Half of the mice were sacrificed 0.5 hours after the 12th injection, and the other half were sacrificed 48 hours after the 11th injection. At the indicated times, tissues from 4 mice were pooled and homogenized for amphotericin B determination.

In another study by Albert *et al.*, 1995 (J Mycol Med 5: 1), ICR mice were infected intracerebrally with *Cr. neoformans* (108 - 800 cfu) and treatment was initiated 48 hours after infection. Different doses of drugs (categorized as low and high dose groups as shown below) were administered by IV route on days 2, 3, 5, 7, 9, 12 and 15 post-infection. Mice were followed for 30 (low dose group) to 60 (high dose group) days post-challenge for survival. Infected, untreated animals were used as controls. No vehicle (i.e., empty liposome)-treated mice were included in the study.

	Dose	**Outcome measure**
Low	0.3 and 1 mg/kg	survival and fungal burden
High	3 and 7 mg/kg	survival
	20 and 30 mg/kg	fungal burden

Results in Figures 8 and 9 show that amphotericin B given as a free drug (≥ 0.3 mg/kg) or as a liposomal preparation (≥ 1 mg/kg) improves survival of infected mice. In the low dose experimental group, free amphotericin B at a dose of 1 mg/kg was more effective in improving survival than the liposomal drug at the same dose (Figure 8). In the high dose experiment, both amphotericin B and AmBisome were equally effective in improving the survival (Figure 9). It is of note that the inocula used for infecting mice in the high dose experiment was half the concentration of that used in the low dose studies (108 vs. 206 cfu).

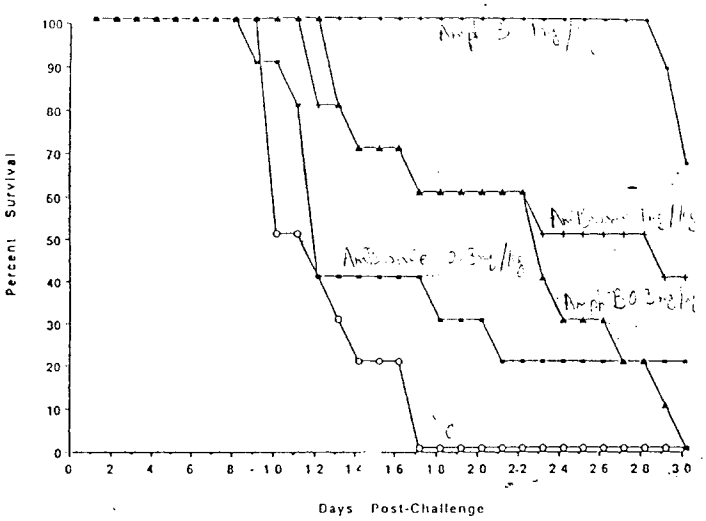


FIG. 8 — Low dose survival experiment. Survival of ICR mice infected intracranially with 206 cfu *C. neoformans* each and treated, iv with AmBisome® or Fungizone®. Infected, untreated controls (○), AmBisome® 0.3 mg/kg/dose (■), Fungizone® 0.3 mg/kg/dose (▲), AmBisome® 1.0 mg/kg/dose (+), Fungizone® 1.0 mg/kg/dose (♦).

Survie des souris traitées par de faibles doses d'antifongiques. La survie de souris ICR infectées par l'injection intracranienne de 206 UFC de *C. neoformans* et traitées par voie intraveineuse avec l'amphotéricine B. Souris infectées non traitées (○), traitées par AmBisome® à 0,3 mg/kg (■), Fungizone® à 0,3 mg/kg (▲), AmBisome® à 1 mg/kg (+) et Fungizone® à 1 mg/kg (♦).

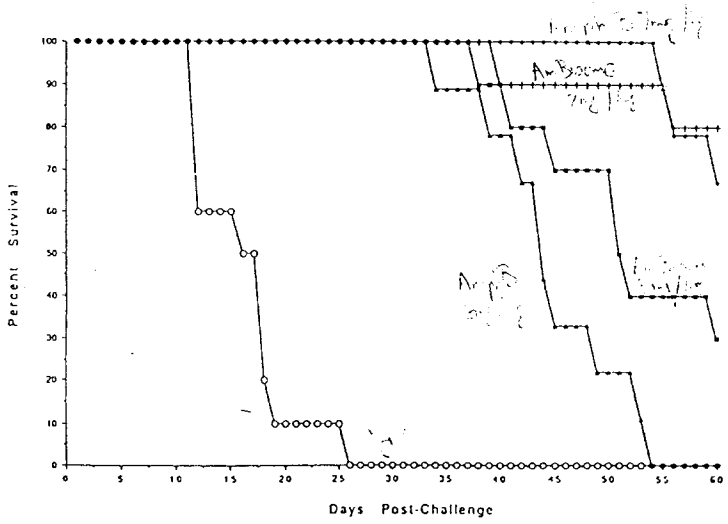


FIG. 9 — High dose survival experiment. Survival of ICR mice infected intracranially with 108 cfu *C. neoformans* each and treated, iv with AmBisome® or ip with Fungizone®. Infected, untreated controls (○), AmBisome® 3.0 mg/ kg/dose (■), Fungizone® 3.0 mg/kg/dose (▲), AmBisome® 7.0 mg/kg/dose (+), Fungizone® 7.0 mg/kg/dose (♦).

Survie des souris traitées par des doses élevées d'antifongiques. La survie de souris ICR infectées par l'injection intra-cranienne de 108 UFC de *C. neoformans* et traitées par voie intraveineuse avec l'AmBisome® ou i. p. avec la Fungizone®. Souris infectées non traitées (○), traitées par AmBisome® à 3,0 mg/kg (■), Fungizone® à 3,0 mg/kg (▲), AmBisome® à 7,0 mg/kg (+) et Fungizone® à 7,0 mg/kg (♦).

Fungal burden in the brain was determined on day 11 (i.e., 2 days after discontinuation of treatment), and day 37 (i.e., about 22 days after discontinuation of therapy) post-infection in mice from the low dose experiment and on day 30 post-infection in mice from the high dose experiment. Mice were infected with 165 or 161 cfu of *Cr. neoformans*. Results in Figures 10 and 11 show that amphotericin B (≥ 0.3 mg/kg) and AmBisome (≥1 mg/kg) were effective in reducing the fungal burden in the brain compared to the untreated controls. On day 11, the activity of free amphotericin B was shown to be superior to the liposomal preparation. Fungal burden in the high dose treated groups (> 3 mg/kg) was not measured. Also of note is that cfu count was higher on day 37 as compared to day 11 thereby showing relapse of infection once treatment is discontinued. This relapse was observed in the groups treated with free amphotericin B or the liposomal preparation.

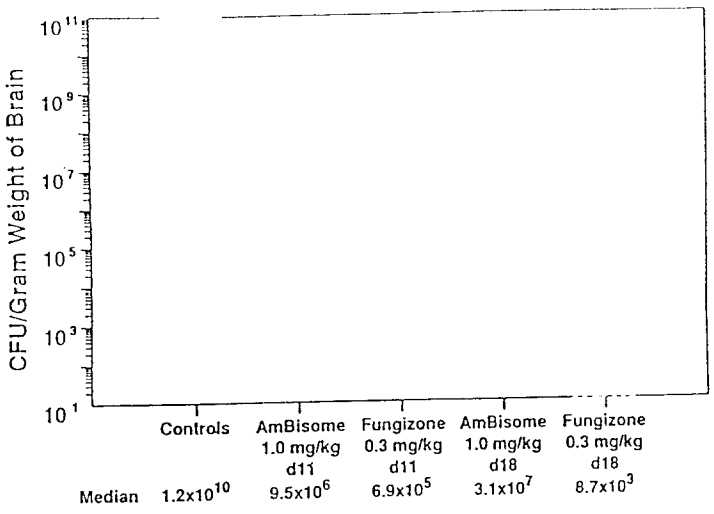


FIG. 10 — Low dose brain tissue counts. Counts determined day 11 and day 18 post-challenge. ICR mice were infected intracranially with 156 cfu *C. neoformans* each and treated with low doses of AmBisome® or Fungizone®. Infected, untreated controls (o), AmBisome® 1.0 mg/kg/dose (▵), Fungizone® 1.0 mg/kg/dose (♦).

Décomptes des cryptocoques dans le cerveau des souris traitées par de faibles doses d'antifongiques. Les numérations ont été effectuées à J11 et J18 après inoculation intracérébrale de souris ICR avec 156 UFC de *C. neoformans*. Souris infectées non traitées (o), traitées par AmBisome® à 1 mg/kg (▵) ou Fungizone® à 1 mg/kg (♦).

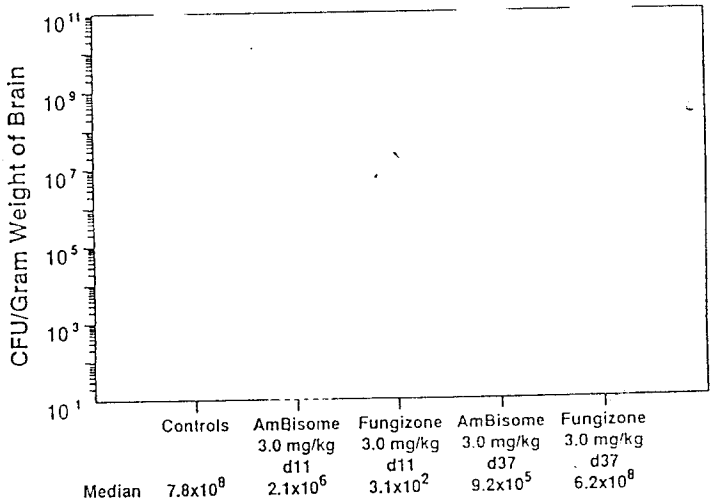


FIG. 11 — High dose brain tissue counts. Counts determined day 11 and day 37 post-challenge. ICR mice were infected intracranially with 161 cfu *C. neoformans* each and treated with higher doses of AmBisome® or Fungizone®. Infected, untreated controls (o), AmBisome® 3.0 mg/kg/dose (▵), Fungizone® 3.0 mg/kg/dose (♦).

Décomptes des cryptocoques dans le cerveau des souris traitées par des doses élevées d'antifongiques. Les numérations ont été effectuées à J11 et J37 après inoculation intracérébrale de souris ICR avec 161 UFC de *C. neoformans*. Souris infectées non traitées (o), traitées par AmBisome® à 3 mg/kg (▵) ou Fungizone® à 3 mg/kg (♦).

In another experiment, mice were infected with 800 cfu of *Cr. neoformans* by the intracranial route and treated with AmBisome (20 mg/kg or 30 mg/kg) IV. Results in Figure 12 show a greater reduction in cfu 2 weeks after discontinuation of therapy than was observed in the above experiment despite the fact that the concentration of the inocula used for infection had a > 4 fold higher cfu count. The untreated mice had a median count of 3.7×10^9 cfu/g brain tissue which is in the range shown above (Figures 10 and 11). Also, of note is the fact that 4/9 (44%) and 7/9 (78%) of the brains from infected mice treated with the 20 and 30 mg/kg dose, respectively, were culture negative.

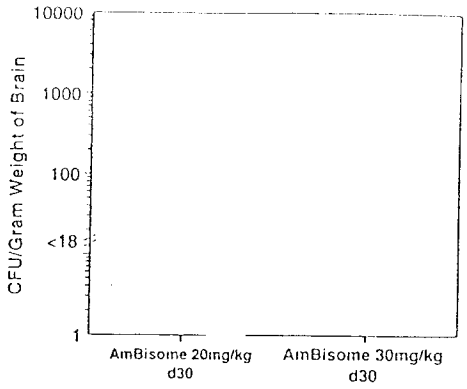


FIG. 12 — Dose tolerance/relapse prevention. Counts determined day 30 post-challenge. Mice were infected intracranially with 800 cfu *C. neoformans* each and treated iv with AmBisome®, 20 mg/kg/dose (o), 30 mg/kg/dose (■).

E. *Blastomyces dermatitidis*

CD-1 mice were infected with ATCC isolate (26199) of *B. dermatitidis* (1.9 or 2.7 x 10^4 cfu) organisms by the intranasal route (Clemons and Stevens, 1993, J Antimicrob Chemother 32: 465). Beginning 4 days after infection, mice were treated 3 times a week for 2 weeks with different doses of either amphotericin B (injected IP) or AmBisome (administered IV). The results show that amphotericin B (1 mg/kg) or AmBisome (≥ 1 mg/kg) was effective in improving survival (Figures 13 and 14) compared to the untreated control. Fungal burden in the lungs of infected mice was also shown to be reduced in a dose dependent manner (Tables 23 and 24). However, a group treated with empty liposomes or vehicle was not included in the study.

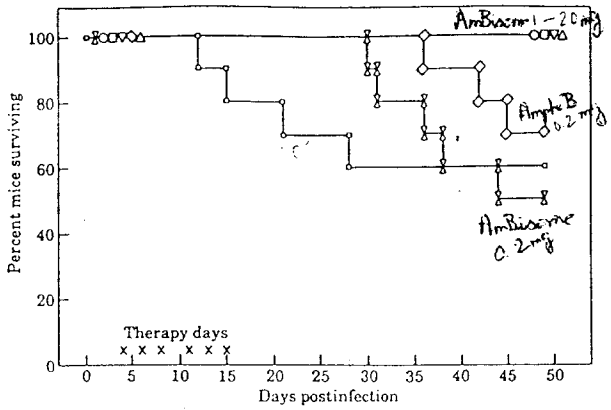


Figure 13 Cumulative survival of mice infected with 19,400 cfu of *B. dermatitidis* and given the various treatment regimens. Untreated controls (□); AmBisome: 0·2 mg/kg (§), 1·0 mg/kg (○), 5·0 mg/kg (□), 7·5 mg/kg ▽; Fungizone 0·2 mg/kg (◇), 1·0 mg/kg (△).

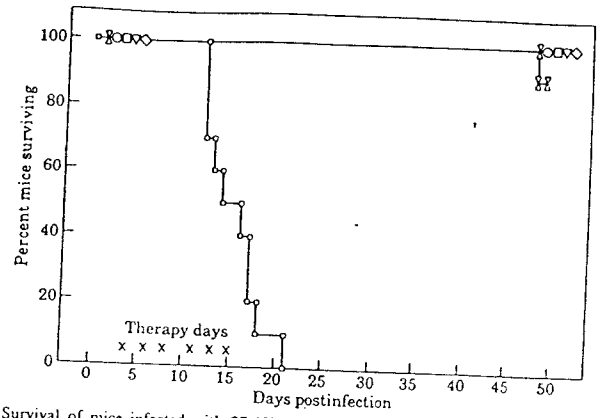


Figure 14 Survival of mice infected with 27,450 cfu of *B. dermatitidis* and given the various treatment regimens. Untreated controls (□); AmBisome: 1·0 mg/kg (§), 3·0 mg/kg (○), 7·5 mg/kg (□), 15·0 mg/kg (▽); fungizone 1·0 mg/kg (◇).

Table 23 Recovery of *B. dermatitidis* from the lungs of surviving mice infected with 19,300 cfu and treated with various doses of Fungizone, AmBisome or untreated controls

Group and dose (mg/kg)	Number of surviving mice	Number of mice cleared of infection	Geometric mean $\log_{10}$ cfu/lung in surviving mice	Range $\log_{10}$ cfu
Untreated	6	3	2·13	
Fungizone				
0·2	7	1	4·85	
1·0	10	1	2·12	
AmBisome				
0·2	5	2	1·88	
1·0	10	0	5·45	
5·0	10	3	1·32	
20·0	10	10	0	

Table 24 Recovery of *B. dermatitidis* from the lungs of surviving mice infected with 27,450 cfu and treated with various doses of Fungizone, AmBisome or untreated controls

Group and dose (mg/kg)	Number of surviving mice	Number of mice free of infection	Geometric mean $\log_{10}$ cfu/lung in surviving mice	Range $\log_{10}$ cfu
Untreated	0	0	—	
Fungizone				
1·0	10	0	3·46	
AmBisome				
1·0	9	0	6·53	
3·0	10	1	3·42	
7·5	10	8	0·22	
15·0	10	7	0·42	

F. *Coccidioides immitis*

ICR mice were infected by the intranasal route with about 200 arthroconidia of *C. immitis* (Albert *et al.*, 1994, J Medical Vet Mycology 32: 467). Treatment with amphotericin B or AmBisome was initiated 3 days post-infection. Additional doses were administered on days 5, 7, 9, 12 and 15 post-infection. Survival was followed for 30 days post-challenge and then the remaining animals were sacrificed and lung lobes weighed. Earlier studies by Huppert *et al.*, 1976 (Inf Immun 14: 1356) demonstrated that a challenge with 200 arthroconidia of *C. immitis*, if left untreated, lead to death in 30% of the mice within a month. Also, lung weight was shown to be greater in mice which succumbed to infection as compared to the surviving mice (the lungs were weighed in all the surviving animals after completion of 30 days of observation). These authors did not provide the survival data. Results in Table 25 show that AmBisome and amphotericin B at a dose of $\geq$ 0.3 mg/kg were effective in reducing the lung weight. The fungal burden or histopathological examination of the tissues was not conducted. Therefore, the possibility of a reduction in lung weight due to a reduction in the inflammatory response or other factors cannot be ruled out.

TABLE 25. Median lung weights of ICR mice infected with *C. immitis* and treated, IV, with various doses of Fungizone or AmBisome

Days post-challenge	Treatment and dose (mg kg^{-1})	Experiment 1		Experiment 2	
		n	median lung weight (g) (range)	*n*	median lung weight (g) (range)
15	Infected controls	—	ND	6	0·7742
	Uninfected controls	—	ND	5	0·2503
	Fungizone 0·3	—	ND	5	0·2660
	AmBisome 0·3	—	ND	5	0·2554
	Fungizone 1·0	—	ND	4	0·2402
	AmBisome 1·0	—	ND	5	0·2766
22	Infected controls	—	ND	4	0·8679
	Uninfected controls	—	ND	5	0·2489
	Fungizone 0·3	—	ND	5	0·2611
	AmBisome 0·3	—	ND	5	0·2775
	Fungizone 1·0	—	ND	4	0·2520
	AmBisome 1·0	—	ND	5	0·2505
30	Infected controls	10	0·8933 (0·6994–0·8933)	9	0·8967
	Uninfected controls	9	0·2430 (0·1300–0·2674)	9	0·2430
	Fungizone 0·05	10	0·8933 (0·3876–0·8933)	—	ND
	AmBisome 0·05	10	0·8933 (0·3692–0·8933)	—	ND
	Fungizone 0·3	9	0·2817 (0·2412–0·8933)	10	0·2660
	AmBisome 0·3	10	0·3002 (0·2713–0·8933)	5	0·2444
	Fungizone 1·0	—	ND	8	0·2651
	AmBisome 1·0	—	ND	10	0·2855

ND = not done.

G. *Paracoccidioides brasiliensis*

Balb/c mice were infected with *P. brasiliensis* (4.1 x 10^7 cfu) by the intranasal route and treatment with AmBisome or amphotericin B was initiated 24 hours after infection by the same route, 3 times a week for 2 weeks (Clevens and Stevens , 1993 J Med Vet Mycol 31: 387). Animals treated with empty liposomes were used as a control. Mice were followed for a period of 44 days post-infection. All the surviving mice were sacrificed and lungs were processed for determination of fungal burden. Results in Figure 15 show that all the untreated mice died by 11 days of infection. AmBisome at a high dose of 27 mg/kg was effective in improving the survival of 80% of the infected mice. AmBisome or amphotericin B at a dose of 1 mg/kg was less effective. Amphotericin B at 1 mg/kg was toxic. Although fungal burden was reduced in mice treated with high dose AmBisome (27 mg/kg) or amphotericin B (1 mg/kg) compared to untreated animals and other dose groups, none of the mice were cleared of the infection (Table 26). A statistical analysis of the data was not done.

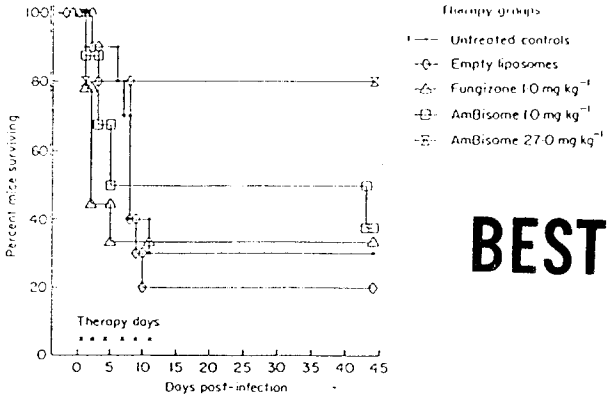


FIG. 15. Cumulative mortality of mice infected with *P. brasiliensis* and given one of the various therapy regimens or no treatment (experiment 1).

TABLE 26. Residual lung burdens of *P. brasiliensis* in surviving mice in experiment 1

Group (no. of animals)	No. cured/ No. survivors	Geometric mean $\log_{10}$ CFU in survivors	Range $\log_{10}$ CFU in survivors
Untreated (10)	0/3	6·88	
Empty liposomes (10)	0/2	6·85	
Fungizone			
1·0 mg kg^{-1} (9)	0/3	4·8	
AmBisome			
1·0 mg kg^{-1} (8)	0/3	6·86	
27·0 mg kg^{-1} (10)	0/8	3·18	

In another experiment mice were infected with 1.6×10^7 cfu of the same isolate of *P. brasiliensis* and treated as described above. Results in Figure 16 show that AmBisome at doses of ≥ 5 mg/kg was effective in improving the survival. AmBisome at a 15 mg/kg dose was most effective in improving survival (86%), but amphotericin B at a dose of 0.6 mg/kg was less effective (47% survival). AmBisome at a dose of ≥ 5 mg/kg was also effective in reducing the fungal burden in the lung (Table 27). At higher doses i.e., 15 and 30 mg/kg AmBisome was effective in clearing fungal organisms from the lung of 36% and 70% of the surviving mice, respectively.

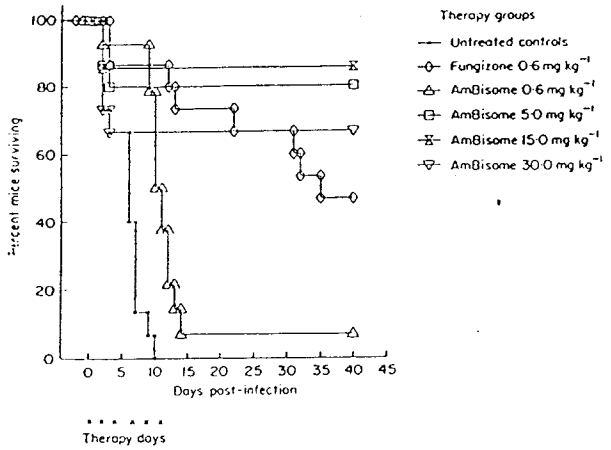


FIG. 16. Cumulative mortality of mice infected with *P. brasiliensis* and given one of the various therapy regimens or no treatment (experiment 2).

TABLE 27. Residual lung burdens of *P. brasiliensis* in surviving mice in experiment 2

Group (no. of animals)	No. cured/ No. surviving	Geometric mean $\log_{10}$ CFU in survivors	Range $\log_{10}$ CFU in survivors
Untreated (15)	0/0	—	
Fungizone			
0·6 mg kg^{-1} (15)	0/7	7·11	
AmBisome			
0·6 mg kg^{-1} (14)	0/1	4·02	
5·0 mg kg^{-1} (15)	0/12	5·09	
15·0 mg kg^{-1} (14)	4/11	1·25	
30·0 mg kg^{-1} (15)	7/10	0·56	

H. *Histoplasma capsulatum*

Congenitally athymic *nu/nu* mice (from a Balb/c background) were infected with *H. capsulatum* (strain G217 at 5 x 10^4 cfu/mouse and isolate 93-255 at 3 x 10^6 cfu/mouse) and treatment with AmBisome or amphotericin B was administered IV (except, at a dose higher than 1 mg/kg AmBisome was administered intraperitoneally) on days 2, 3, 5, 9, 12 and 15 post-infection (Graybill and Bocanegra, 1995, Antimicrob Agents Chemother 39: 1885). Mice were followed for survival for a period of 40 days after infection. AmBisome (at a dose of ≥ 1 mg/kg) and amphotericin B (at a dose of 0.3 mg/kg) were effective in prolonging the survival of mice infected with the G217 or 93-255 strains of *H. capsulatum* (Table 28). The survival time varied with the virulence of the strain of *H. capsulatum* used for infection.

In another experiment mice were infected with isolate 93-255 (3 x 10^6 cfu/mouse) and treatment with AmBisome or amphotericin B was administered on days 2, 3, 5 and 7 post-infection. Mice were sacrificed 2 days after discontinuation of treatment and spleens and kidneys were processed for measurement of fungal burden. Results in Figure 17 show that AmBisome and amphotericin B at a dose of 1 mg/kg were effective in reducing fungal burden in spleen. In the kidney, amphotericin B at a dose of 1 mg/kg was more effective than AmBisome at the same dose. The lower dose (0.6 mg/kg) was less active. Higher doses were not tested.

TABLE 28 Survival of mice with disseminated histoplasmosis treated with AmBisome or amphotericin B

Isolate and group	Dose (mg/kg)	Mean days survival	SEM*
G217			
Control	None	29	2.1
Ampho B	0.3	37	4.0
	1.0	46	—**
	3.0	37	3.6
AmBisome	0.3	30	3.1
	1.0	28	3.9
	3.0	46	—**
93-255			
Control	None	6	0.2
Ampho B	0.3	9	0.9*
	0.6	14	2.7*
AmBisome	0.3	9	0.2*
	0.6	14	1.6*
None			
Control	None	7	0.1
Ampho B	1.0	10	0.9**
	3.0	23	2.9**
AmBisome	1.0	16	2.5***
	3.0	29	0.8**

* —, none (all mice survived the study); *, $P < 0.005$ compared with control; **, $P < 0.001$ compared with controls; ***, $P < 0.02$ compared with amphotericin B (Ampho B) at 1.0 mg/kg, and <0.001 compared with control.

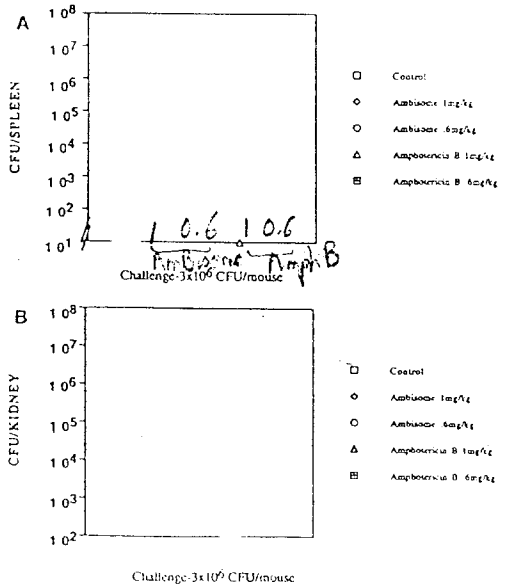


FIG 17 Tissue counts for the spleens (A) and kidneys (B) of mice infected with 3×10^6 CFU of *H. capsulatum* 93-255 and treated until day 7. The mice were sacrificed on day 9. AmBisome is the trade name of liposomal amphotericin B.

I. *Trichosporon beigelii*

CF1 mice were immunosuppressed with a single injection of cyclophosphamide (150 mg/kg) IP, 24 hours before IV infection with 5 x 10^6 cfu of *T. beigelli* (Anaissie *et al.*, 1994 Antimicrob Agents Chemother 38: 2541). Starting at one hour post-infection, mice were treated with AmBisome or amphotericin B once daily for 10 days. Four days after infection, 5 of the mice from each group were sacrificed and kidneys processed for measurement of fungal burden. The authors have stated that liposomal amphotericin B did not improve the survival of infected mice (data not shown). However, the fungal burden was marginally reduced by treatment with either amphotericin B or AmBisome (Table 29). The activity of the two formulations of the drug were comparable. It is also of note that the observed activity was under experimental conditions wherein immunosuppression was induced by a single injection of cyclophosphamide. The possibility exists for the drug to be less effective if immunosuppression is maintained throughout the period of observation.

TABLE 29 Effects of amphotericin B and liposomal amphotericin B on clearance of fungi from kidneys of mice with disseminated trichosporonosis

Treatment group (dose [mg/kg/day])	Isolate 009		Isolate 008	
	Mean ± SE $\log_{10}$ CFU/g of kidney tissue[a]	*P* value vs no therapy	Mean ± SE $\log_{10}$ CFU/g of kidney tissue[a]	*P* value vs no therapy
No therapy	5.93 ± 0.33		6.87 ± 0.18	
Amphotericin B (1)	5.75 ± 0.04	NS[b]	5.88 ± 0.13	<0.05
Liposomal amphotericin B				
1	6.05 ± 0.08	NS	6.27 ± 0.11	<0.05
5	5.66 ± 0.39	NS	6.13 ± 0.07	<0.05
10	5.38 ± 0.35	<0.05	5.76 ± 0.23	<0.05

[a] Results represent data from three experiments with five mice in each experimental group.
[b] NS, not statistically significant.

II. ANTI-PARASITIC ACTIVITY

The sponsor has submitted studies to show the activity of AmBisome against the amastigote stage of the protozoan parasite, *Leishmania*. *Leishmania* is a hemoflagellate, intracellular parasite which resides within the phagolysosome. There are at least 3 species of this parasite which are considered to be the causative agent for visceral leishmaniasis. These include *L. donovani*, *L. infantum* and *L. chagasi*. The sponsor has submitted studies demonstrating activity *in vivo* against 2 of the *Leishmania* species i.e., *L. donovani*, and *L. infantum*.

The promastigote stage of *Leishmania* is transmitted to humans when bitten by an infective sandfly. Once engulfed by the cells of the reticuloendothelial system, the promastigotes transform to amastigotes. The amastigote is an intracellular stage which can resist the antimicrobial effects of the oxidative burst and survives and multiplies asexually within the phagolysosome of the phagocytic cells.

Studies *in vitro*

Peritoneal macrophages obtained from mice were infected with *L. donovani* amastigotes (isolated from hamsters - concentration not specified) in RPMI 1640 medium containing 10% heat inactivated fetal calf serum at 37°C in 5% CO_2. Cultures were exposed to different concentrations of AmBisome or amphotericin B either once or 3 times during the 7 day period of observation (Croft *et al.*, 1991, J Antimicrob Chemother 28 (Suppl B): 111). The proportion of infected macrophages was determined by microscopic examination of Giemsa stained smears. Results in Table 30 show that both amphotericin B and AmBisome were effective in reducing the percentage of infected macrophages. The actual parasite count or the growth of the parasite over time was not measured. It is not clear from the publication how long after initiation of infection the drugs were added to the cultures. It is possible that both inoculum used for infection and drugs may have been added simultaneously. Whether the activity of the drug will be altered in chronically infected macrophages is not known.

Table 30 The activity of AmBisome and conventional amphotericin B against *L. donovani* amastigotes in mouse peritoneal macrophages in-vitro

Formulation, dose (mg/L), no of doses		Infected macrophages (±S.E.M.)	% suppression
Untreated controls		79·75 (±1·60)	
Conventional amphotericin B	0·1 (×3)	0	100·00
	0·01 (×3)	0·75 (±0·48)	99·0
Conventional amphotericin B	0·1 (×1)	0	100·00
	0·01 (×1)	22·25 (±2·90)	67·30
AmBisome	0·1 (×3)	0	100·00
	0·01 (×3)	14·75 (±2·59)	80·90
AmBisome	0·1 (×1)	1·25 (±0·75)	98·40
	0·01 (×1)	41·75 (±3·12)	46·00
Blank liposomes*	(×3)	75·75 (±2·96)	1·94

*Provided by Vestar; diluted to phospholipid level equivalent of 0·5 mg/L AmBisome dose.

The authors have stated that AmBisome was not active against the promastigote stage of the parasite (free living stage which is transmitted to humans by the bite of infective sandfly). However, details of the experimental design and results were not included in the report.

Studies *in vivo*

A. *Leishmania donovani*

Balb/c mice were infected IV with 5 x 10^6 amastigotes of *L. donovani* harvested from the spleen of infected hamsters (Croft *et al.*, 1991, J Antimicrob Chemother 28 (Suppl B): 111). A week after infection, mice were treated with a single dose of AmBisome or amphotericin B IV. The activity of the drug was measured by counting the number of amastigotes in the liver on day 8. The actual parasite count was not provided. The results in Table 31 show that AmBisome at a single dose of ≥ 5 mg/kg was effective in inhibiting parasitic burden in the liver. Amphotericin B up to a dose of 1 mg/kg was less effective. Empty liposomes did not exhibit any antiparasitic activity.

Table 31 The activity of single doses of AmBisome and conventional amphotericin B against *L. donovani* amastigotes in the liver of BALB/c mice

Formulation	Dose (mg/kg)	% inhibition	(P value)	Activity values[a] (mg/kg) (P95 limits)
Experiment 1				
AmBisome	25·0	99·2	(0)	$ED_{\infty} = 0{\cdot}154$
	5·0	99·9	(0)	
	1·0	85·4	(0)	$ED_{50} = 2{\cdot}653$
	0·25	53·8	(0)	
Conventional amphotericin B	1·0[b]	42·2	(0·0002)	$ED_{\infty} = 4{\cdot}914$
	0·2	14·1	(0·0308)	
	0·05	5·3	(0·256)	
Blank liposome[c]	—	8·25	(0·135)	—
Experiment 2				
AmBisome	5·0	99·8	(0)	$ED_{\infty} = 0{\cdot}256$
	1·0	84·5	(0)	
	0·2	41·2	(0·001)	$ED_{50} = 2{\cdot}253$
	0·04	15·8	(0·107)	
Conventional amphotericin B	1·0[b]	52·7	(0·0003)	$ED_{50} = 0{\cdot}951$
	0·2	22·0	(0·0161)	
	0·04	3·4	(0·688)	

[a] ED_{∞} values for amphotericin B outside tolerated range of formulation.
[b] Toxicity to mice observed following drug administration.
[c] Provided by Vestar Inc.; diluted to phospholipid level equivalent of 25·0 mg/kg AmBisome dose.

The activity of the drugs was also measured after multiple dosing with AmBisome or amphotericin B on days 7 and 11 or days 7, 9 and 11. The parasitic load was measured in the liver and spleen 2 weeks post-infection (i.e., 3 to 4 days after discontinuation of treatment). Results in Table 32 show that AmBisome at a dose of 0.2 mg/kg, administered 2 (cumulative dose 0.4 mg/kg) or 3 times (cumulative dose 0.6 mg/kg) was less effective than free amphotericin B. This may be due to the very low dose of AmBisome used in comparison to amphotericin B. Nevertheless this experiment does show that the antiparasitic activity of AmBisome against *L. donovani* is less in spleen compared to liver. The reasons for this are not clear. The actual parasitic count in treated and untreated mice was not included in the publication. However, the authors have stated that the level of infection achieved in the spleen was considerably lower than in the liver.

Table 32 The activity of multiple doses of AmBisome and conventional amphotericin B against *L. donovani* amastigotes in the liver and spleen of BALB/c mice

Formulation	Dose	% Inhibition liver	(*P* value)	% Inhibition spleen	(*P* value)
AmBisome	0·2 mg/kg				
	× 3	59·8	(0·000)	47·5	(0·0008)
	× 2	48·2	(0·0001)	23·7	(0·0672)
	× 1	36·0	(0·0001)	6·96	(0·3318)
Conventional amphotericin B	1·0 mg/kg				
	× 3	73·3	(0·000)	58·5	(0·000)
	× 2	71·5	(0·000)	47·8	(0·000)
	× 1	56·9	(0·000)	27·2	(0·0750)
Blank liposomes[a]					
	× 3	14·9	(0·126)	15·5	(0·1272)

[a] Provided by Vestar Inc.; diluted to phospholipid equivalent of 25 mg/kg AmBisome dose.

Another study was conducted by Berman *et al.*, 1986 (Antimicrob Agents Chemother 30: 847) in hamsters and monkeys. Hamsters were infected with 10^7 amastigotes of *L. donovani* by the intracardiac route. Treatment with a single dose of AmBisome, amphotericin B, meglumine antimonate, liposomal meglumine antimonate or free liposome was initiated 3 or 10 days post-infection. All drugs except liposomal meglumine antimonate (administered intramuscularly) were administered by the intracardiac route. Four days after treatment, the number of parasites was

enumerated in liver and spleen impression smears. Results in Tables 33 and 34 show that AmBisome, free amphotericin B, meglumine antimonate, and liposomal meglumine antimonate exhibited activity against the amastigote stage in the liver and spleen. Such an effect was observed in both the models when treatment was initiated 3 or 10 days post-infection. However, the activity of the drug was better when treatment was initiated at 3 days as compared to 10 days post-infection when the animals would have a more severe infection. It should be noted that parasitic load was measured 4 days after discontinuation of treatment. Studies in mice have shown significant levels of amphotericin B in spleen, liver and other tissues, even 2 weeks after discontinuation of treatment with liposomal amphotericin B. Therefore, the observed parasitic effect could be due to the continued presence of drug in the tissues and infection may relapse once the drug is eliminated.

TABLE 33 Activity of liposomal preparations against *L. donovani* in hamsters infected for 3 days[a]

Drug	Dosage (mg/kg)	Liver amastigotes			Spleen amastigotes		
		% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{50}/G_{90}	% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{90}
Sb[b]	416	99 ± 0.4	75/232	1/1	91 ± 7	—[c]/≅416	1
	104	77 ± 3			61 ± 10		
	52	17 ± 7			72 ± 9		
	13	3 ± 11			0		
Liposomal Sb[b]	50	99 ± 0	—/0.3	—/933	99 ± 0.5	—/0.5	832
	12.5	99 ± 0			99 ± 0.3		
	3.2	99 ± 0.3			98 ± 1		
	0.8	99 ± 0.9			98 ± 1		
	0.2	86 ± 3			83 ± 10		
AmB	6.0	99 ± 0	≅0.4/≅1.5	187/155	99 ± 0.2	0.3/3.2	130
	1.5	92 ± 2			84 ± 9		
	0.4	54 ± 5			67 ± 9		
	0.1	21 ± 12			0		
L-AmB	6.0	99 ± 0	≅0.1/0.7	750/331	99 ± 0.5	—/1.0	416
	1.5	99 ± 0.5			99 ± 0.1		
	0.4	70 ± 5			80 ± 9		
	0.1	48 ± 2			72 ± 7		
Liposomes		0			3 ± 16	—/—	—

[a] In control animals, there was a mean ± standard error of 743 × 10^6 ± 48 × 10^6 parasites per liver and 6.5 × 10^8 ± 1.3 × 10^8 parasites per spleen.
[b] In the form of Glucantime.
[c] —, Not determined.

TABLE 34 Activity of liposomal preparations against *L. donovani* in hamsters infected for 10 days[a]

Drug	Dosage (mg/kg)	Liver amastigotes			Spleen amastigotes		
		% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{50}	% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{50}
Sb[b]	416	68 ± 5	208/>416	1	75 ± 5	188/>416	1
	208	46 ± 10			57 ± 13		
	104	21 ± 7			20 ± 12		
	52	0			25 ± 8		
Liposomal Sb[b]	28	100	0.20/0.8	1,040	100	0.20/0.07	940
	7	99 ± 0			99 ± 0.1		
	1.4	99 ± 0.1			99 ± 0.3		
	0.28	83 ± 3			85 ± 5		
	0.07	4 ± 9			7 ± 11		
AmB	6	78 ± 2	≅1.5/—[c]	139	60 ± 4	4.4/—	43
	1.5	48 ± 3			31 ± 10		
	0.4	5 ± 8			26 ± 10		
L-AmB	11	99 ± 0.05	1.0/2.7	208	99 ± 0	1.1/≅2.8	170
	2.8	93 ± 1.6			91 ± 2		
	0.7	42 ± 4			40 ± 10		
	0.17	21 ± 9			11 ± 19		
	0.04	5 ± 5			0		
Liposomes		0			29 ± 14		

[a] In control animals, there was a mean ± standard error of 1,790 × 10^6 ± 128 × 10^6 amastigotes per liver and 46 × 10^8 ± 4.8 × 10^8 amastigotes per spleen.
[b] In the form of Glucantime.
[c] —, Not determined.

Squirrel monkeys were infected with 5 x 10^8 amastigotes of *L. donovani* IV. Treatment with multiple doses of AmBisome, amphotericin B, meglumine antimonate, liposomal meglumine antimonate or free liposome was initiated 17 days post-infection. All drugs except liposomal meglumine antimonate (administered intramuscularly) were administered by the intracardiac route. Four days after discontinuation of treatment, the number of parasites was enumerated in liver and spleen impression smears. Results in Table 35 show that AmBisome was effective in reducing the parasite count in liver and spleen. The drug free liposome also showed some activity against the parasite. However, authors have attributed this apparent activity to gender differences. Both of the liposome treated monkeys were females whereas the rest of the animals used in the study were males.

TABLE 35 Activity of L-AmB against *L. donovani* infection in squirrel monkeys[a]

Drug	Dosage	No. of monkeys dead/total (day[s] of death)	% Suppression [mean (range)]	
			Liver amastigotes	Spleen amastigotes
Sb[b]	104 mg/kg per day × 7 days (728 mg/kg)	1/3 (9)	98 (98–100)	99 (99–100)
AmB	2 mg/kg on days 1, 4, and 7 (6 mg/kg)	2/2 (2, 9)	96 (95–97)	98 (96–100)
L-AmB	2 mg/kg on days 1, 4, and 7 (6 mg/kg)	0/3	95 (88–100)	90 (89–92)
	4 mg/kg on day 1 (4 mg/kg)	0/3	90 (85–100)	71 (67–76)
	4 mg/kg on days 1, 4, and 7 (12 mg/kg)	1/3 (10)	99 (98–100)	98 (99–100)
Liposomes		0/2[c]	73 (42–89)	57 (44–61)

[a] In the three control animals, there was a mean (range) of 1.1 (1.04 to 1.12) × 10^9 organisms per liver and 27 (18 to 38) × 10^6 organisms per spleen.
[b] In the form of Glucantime.
[c] This group comprised female monkeys.

(b) *Leishmania infantum*

Balb/c mice were infected with a semipurified suspension of amastigotes (3 x 10^6) IV (Gradoni *et al.*, 1993 J Drug Targeting 1: 311). Treatment with AmBisome (group III - days 1, 2, and 3; group V - days 1, 2, 3, 5 and 10; and group VII - days 1, 2, 3, 5, 10, 18 and 25) or meglumine (Sb - days 1 to 21) by subcutaneous and intravenous route, respectively, was initiated on day 12 post-infection for different time intervals. Mice were sacrificed at different time intervals and an amastigote count was performed on liver smears. Spleen was not tested. Results in Figure 18 and Table 36 show AmBisome to be effective in reducing the parasitic load in the liver. Such an effect was observed by 5 days of treatment.

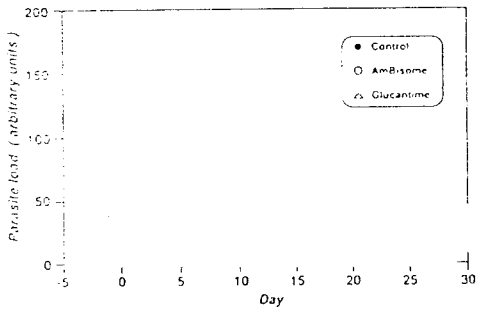


FIGURE 18 Activity of AmBisome and meglumine antimoniate (Glucantime) against *L. infantum* in the liver of BALB/c mice.

TABLE 36
Activity of AmBisome and meglumine antimoniate (Glucantime) against L. infantum in the liver of BALB/c mice[a]

Day	Control	Group III	Group V	Group VII	Group Sb
0	74.2±5.6	—	—	—	—
5	111.8±9.7	0(100)	—	—	27.3±2.5 (75.6)
11	164.3±5.5	0(100)	0(100)	—	8.2±1.3 (95.0)
21	49.3±4.7	0(100)	0(100)	0(100)	2.4±0.8 (95.2)
28	48.2±2.9	0(100)	0(100)	0(100)	0(100)

[a] The values are expressed as mean arbitrary units of parasite load ± SE. Percentage parasite suppression in parentheses

In another study, the activity of AmBisome was measured in dogs naturally infected with *L. infantum* and showing viscerocutaneous signs of disease (Oliva *et al.*, 1995, J Antimicrob Chemother 36: 1013). The animals with clinical signs of disease tested positive for serum antibodies by immunofluorescent antibody test and also showed evidence of parasites in lymph node or bone marrow aspirates. Infected dogs were treated with different doses (a total of 3 to 5 doses) of AmBisome over a period of 3 to 10 days. The dosing regimen was as follows:

Group	Number	Days	Each Dose (mg/kg/day)	Total dose (mg/kg/day)
Group I	3	3 consecutive daily doses	1.67	5
Group II	2	3 consecutive daily doses	3.3	10
Group III	5	1,2, 3 and 10	3.0	12
Group IV	3	1,2,3,4 and 10	3.0	15

The animals were examined for clinical, hematological, biochemical and parasitological outcome at different time intervals over a period of 8 months. The results in Figure 19 show an improvement in clinical outcome after treatment with AmBisome. Such an improvement was observed after about 2 - 4 weeks of treatment and was more apparent in dogs treated with the 10, 12 or 15 mg/kg dose. However, such a clinical response is transient since relapse occurred in dogs from all the groups (within 2 months in group I and 4 to 6 months in group II, III and IV). No other drug was used for comparison to AmBisome nor was any vehicle control included in the study.

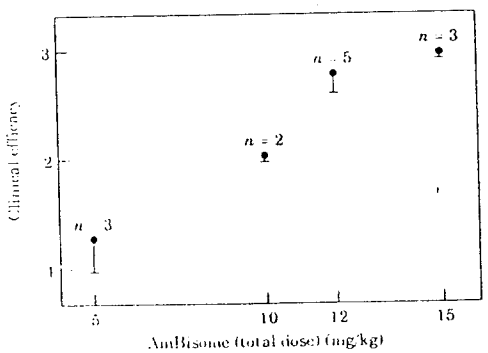


Figure 19: Clinical efficacy of different doses of AmBisome administered to dogs naturally infected with *L. infantum*. Clinical efficacy: mean (with S.E. bar) of the highest clinical efficacy scores shown in the Table.

It was shown that at 2 months post-treatment, lymph node aspirates from all the dogs tested positive for parasite. However, the details of the parasitological observations at baseline, and changes observed during or following discontinuation of treatment were not provided. Nevertheless, the results do show that the continued presence of parasites at 2 months after discontinuation of treatment could be responsible for relapse of infection. The results of the antibody titers during the period of study were not included. However, authors have stated that the antibody titers remained persistently high in 12/13 dogs. It is not clear what class of antibody was measured by the assay used. Such an observation is not unexpected since antigen specific antibodies (especially IgG) once formed persist in circulation for a long period of time and cannot be used as a marker for diagnosis of fresh infection. Antibodies also cannot be used as a measure of drug activity. Of note, antibodies do not play a predominant role in protection against the intracellular phase of *Leishmania*.

All the studies measuring activity of AmBisome against *Leishmania* were conducted in immunocompetent animals. However, the results of studies in naturally infected dogs (Oliva *et al.*, 1995, J Antimicrob Chemother 36: 1013) may reflect the impact of impaired immune function on drug activity since an established *Leishmania* infection can, by itself, cause suppression of the cell-mediated immune response. The immune status of the dogs during the course of study was not measured.

Mechanism of Action

Amphotericin B is a polyene antibiotic with a high affinity for the sterol component of the cell membrane. The binding of this drug with the sterol alters the membrane permeability leading to release of intracellular components (i.e., osmotic imbalances: potassium, proteins, and others) into the extracellular space which results in cell death. Amphotericin B binds to not only fungal cells but also to mammalian cells thus causing severe toxicity. Nevertheless, the affinity of binding to ergosterol (a primary sterol component of the fungal cell membrane) is higher than against cholesterol (a primary sterol component of the mammalian cell membrane).

In addition to alterations in cell membrane permeability, amphotericin B contributes to oxidative cell damage which is independent of the effect of drug on membrane permeability or the nature of sterols. This can cause cell death directly by auto-oxidation. On the contrary, amphotericin B can increase the concentration/activity of the catalase enzyme which can inhibit oxidative cell damage. Another enzyme involved in oxidative cell damage is superoxide dismutase (SOD). Although SOD by itself does not alter the activity of amphotericin B, a combination of catalase with SOD was shown to be synergistic in inhibiting antifungal activity of amphotericin B. Lipid peroxidation, inhibition of membrane enzymes or blockade of endocytosis may also have a role in the second phase of cell death.

The effects of amphotericin B on immune function are less clear. There is evidence to suggest that Amphotericin B may have stimulatory or suppressive effects on cells of the immune system. The

stimulatory response is a part of the oxidative process and is concentration dependent. Low concentrations of amphotericin B (lower than those required to induce permeability changes) increase the phagocytosis of opsonized sheep erythrocytes. This effect can be inhibited by addition of exogenous catalase or SOD enzymes. Higher concentrations did not induce such a stimulatory effect. In contrast, amphotericin B was shown to inhibit differentiation of macrophages *in vitro*.

The mechanism by which amphotericin B exhibits activity against the *Leishmania* parasite was not investigated. However, studies have shown that the major sterol content of the amastigote and/or promastigote stage of *L. tropica*, *L. donovani* and *L. mexicana* is ergosterol, suggesting that the mechanism of action is similar to the anti-fungal drug effects. It is of note that amphotericin B does not exhibit activity *in vitro* against the promastigote stage of the parasite despite the presence of ergosterol. Reasons for this are unclear.

Liposomes can serve as a means for delivering drugs preferentially to cells of the reticuloendothelial system. Some of the drugs such as amphotericin B and nystatin are toxic when administered in free form but have been shown to exhibit reduced toxicity without alteration in their activity when administered as a liposomal preparation (Mehta *et al.*, Biochem Biophys Acta 1984, 770: 230; Lopez-Berestein, G. *et al.*, J Inf Dis 1985, 151: 704; Mehta *et al.*, Antimicrob Agents Chemother 1987, 31: 1897; Mehta *et al.*, Antimicrob Agents Chemother 1987, 31: 1901; Szoka, F. C. Biotechnol Appl Biochem 1990, 12: 496; Fielding, R.M. Clin Pharmacokinetics 1991, 21: 155). However, whether chronic usage of any drug delivered as a liposomal formulation will alter the function of the cells of reticuloendothelial system such as macrophages and neutrophils (which are important components for host defense against *Candida* and other fungal infections) is not known.

AmBisome is a liposomal preparation containing amphotericin B complexed with cholesterol, hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylglycerol (DSPG). Amphotericin B is bound to the lipid component by noncovalent bonds. It is not clear exactly how these bonds break to release free drug. The complex could be broken either by disequilibrium in the constituents of the liposomal preparation or by the action of hydrolytic enzymes (lipases/phopspholipases). These hydrolytic enzymes are present in mammalian cells as well as fungi and are active at acid pH. The distribution of the enzymes within the body appears to be ubiquitous (i.e. present within the lysosomes in phagocytic cells, peroxisomes, plasma etc.). The fate of the different components of the liposomal preparation after dissociation is also not clear. Some of the studies submitted by the sponsor in support of the stability of the liposomal preparation and the specificity of the drug delivery system are discussed below.

Stability of AmBisome in rat plasma

The stability of AmBisome was determined *in vitro* by incubation of the drug in 50% fresh rat plasma for up to 24 hours at 37°C (report # 232-93099). At different time points between the period of 0 to 24 hours, AmBisome was separated from plasma components by size exclusion chromatography using a Recovered AmBisome was analyzed for

individual components of the liposomal preparation which include amphotericin B, cholesterol and hydrogenated soy phosphatidylcholine (HSPC). The results in Table 37 show that incubation for up to 24 hours in rat plasma did not significantly alter the recovery of any of the components of AmBisome analyzed thereby indicating a firm binding between amphotericin B and the lipid.

TABLE 37

Chromatographic Recovery of AmBisome Following 24-Hour Incubation in Plasma

	Total AmB (mg)	Total CHOL (mg)	Total HSPC (mg)	% of stock AmBisome			%AmB: %HSPC
				AmB	CHOL	HSPC	
Stock AmBisome	3.990	3.824	13.820	100%	100%	100%	100%
0 hour incubation	3.600	4.078	14.080	90%	107%	102%	89%
2 hour incubation	3.471	4.627	14.430	87%	121%	104%	83%
8 hour incubation	3.512	4.399	13.280	88%	115%	96%	92%
16 hour incubation	3.370	3.560	11.952	84%	93%	86%	98%
24 hour incubation	3.580	3.810	13.182	90%	100%	95%	94%

Abbreviations: AmB = amphotericin B; CHOL = cholesterol; HSPC = hydrogenated soy phosphatidylcholine.

Effect of AmBisome on Red Blood Cells *in vitro*

The association of AmBisome or Fungizone with cells in freshly collected human whole blood containing EDTA was investigated by measuring the concentration of amphotericin B remaining in plasma (report no. 232-93099). The results show that incubation of whole blood with AmBisome or free amphotericin B for up to 2 hours was associated with 80% and 50% recovery, respectively, thereby indicating higher association of free amphotericin B with cells as compared to AmBisome. Association beyond the 2 hour time point was not investigated.

In another experiment, human red blood cells (RBC) were incubated with either AmBisome (3, 30 and 100 ug/ml) or Fungizone (1, 3 and 6 ug/ml) at 37°C for 15 and 120 minutes. The cells were centrifuged and the hemoglobin released was estimated in the supernatants by measuring the optical density at 541 nm. Results in Table 38 show increased lysis with amphotericin B as compared to AmBisome.

It is of note that although amphotericin B exhibited stable binding during the period of incubation, but the *in vitro* conditions reflect a static environment. These conditions may be very different from the *in vivo* conditions wherein the equilibrium will be more dynamic. In addition, the action of lipases is optimal at acid pH (pH 5), but the *in vitro* experiments were conducted at a neutral pH.

TABLE 38

Effect of AmBisome or Fungizone on
Human Red Blood Cell Lysis

Treatment	Amphotericin B concentration (μg/mL)	Absorbance at 541 nm (% RBC lysis)			
		15 minutes		120 minutes	
Saline control	—	0.005	(1%)	0.006	(1%)
Deoxycholate control	—	0.004	(1%)	0.005	(1%)
Hypotonic shock (100% lysis)	—	0.489	(100%)	0.482	(100%)
AmBisome	3	0.010	(2%)	0.0090	(2%)
	30	0.014	(3%)	0.016	(3%)
	100	0.026	(5%)	0.031	(6%)
Fungizone	1	0.068	(14%)	0.455	(94%)
	3	0.405	(83%)	0.469	(97%)
	6	0.430	(88%)	0.478	(99%)

Also, hydrolytic enzymes like any other enzyme are known to be relatively unstable. The stability of the phospholipases in the *in vitro* experiments remains questionable.

Effect of AmBisome on viability of canine kidney cells and murine macrophages *in vitro*

MDCK (canine kidney) cells and murine macrophages were incubated with AmBisome or amphotericin B for a period of 2 to 6 days and the viability of the cells measured by either MTT assay and/or by incorporation of radio-labeled thymidine, or leucine (report no. 232-93098). The results, expressed as IC_{50} values (50% inhibitory or toxic concentrations), indicate AmBisome to be less toxic (higher IC_{50} values) than free amphotericin B (Tables 39 - 42). The toxicities of AmBisome vs. amphotericin B varied with time of exposure to the drug *in vitro* and the assay used. Also, of note is the fact that macrophages were more sensitive to the toxic effect than kidney cells as indicated by lower IC_{50} values. These toxicities were measured based on incorporation of precursors for synthesis of DNA and proteins or mitochondrial activity. Whether other functions of these cells such as phagocytosis or respiratory burst will be affected by achievable drug levels is not known.

TABLE 39

Kidney Cells - 2 Day Treatment (IC_{50} μg/mL)

Drug	Thy	Leu	MTT
AmBisome	71	134	982
Liposomes w/o drug	925	>1280	1044
Fungizone	30	26	46
Desoxycholate	56	96	92

Abbreviations: Thy = [^{3}H]-thymidine; Leu = [^{3}H]-leucine; MTT = dimethylthiazol diphenyltetrazolium bromide.

TABLE 40

Kidney Cells - 5 Day Treatment (IC_{50} µg/mL)

Drug	Thy	Leu	MTT
AmBisome	378	225	404
Liposomes w/o drug	614	>1280	>1280
Fungizone	73	27	54
Desoxycholate	77	49	55

Abbreviations: Thy = [^{3}H]-thymidine; Leu = [^{3}H]-leucine; MTT = dimethylthiazol diphenyltetrazolium bromide.

TABLE 41

Macrophages - 2 Day Treatment (IC_{50} µg/mL)

Drug	Thy	MTT
AmBisome	18	61
Liposomes w/o drug	846	81
Fungizone	8	6
Desoxycholate	38	39

Abbreviations: Thy = [^{3}H]-thymidine; MTT = dimethylthiazol diphenyltetrazolium bromide.

TABLE 42

Macrophages - 6 Day Treatment (IC_{50} µg/mL)

Drug	Thy	Leu	MTT
AmBisome	66	50	63
Liposomes w/o drug	107	55	75
Fungizone	30	25	< 0.1
Desoxycholate	59	53	< 0.1

Abbreviations: Thy = [^{3}H]-thymidine; Leu = [^{3}H]-leucine; MTT = dimethylthiazol diphenyltetrazolium bromide.

Localization of labeled AmBisome *in vivo* in mice infected with *C. albicans*

Localization of fluorescent labeled AmBisome, empty liposomes or the free dye was investigated in C57BL/6 mice infected with *C. albicans* (2 x 10^6 cfu inoculated IV) using kidney as the target organ (report no. 232-93091). At 23 and 30 hours post-infection, groups of 2 mice were administered either AmBisome or empty liposomes IV labeled with sulforhodamine. Free dye and buffer were included as controls. Mice were sacrificed after 17 hours of treatment with liposomal

preparation (with or without drug) and 7 hours after treatment with the free dye. Serial cryostat sections of the kidneys were processed for examination of the fluorescent stain or examined for pseudohyphae and yeast cells upon staining with Gomori methenamine silver (GMS) stain. The histopathological examination of the kidney sections demonstrated the presence of fungal growth in the kidney of infected mice administered either empty liposomes, free dye or buffer. In mice treated with labeled-AmBisome the staining with GMS was less intense thereby indicating an inhibitory effect of the drug on fungal growth. Examination of the tissues for fluorescence shows localization of the dye in the infected areas in mice treated with empty liposomes as compared to the free dye or the buffer treated mice. Fluorescence staining of kidney sections from mice treated with labeled-AmBisome appears to be less localized as compared to the empty-liposomes treated group but less diffuse than the group treated with free dye or the autofluorescence observed in the buffer treated group. The magnification at which the pictures were taken is not high enough to actually differentiate whether fluorescent staining is at the surface or is cytoplasmic. Whether similar staining/localization will be observed in the kidneys of uninfected mice was not investigated. No comparison was made with the free drug i.e., amphotericin B. Also, other tissues such as liver, spleen, and lymph nodes which are also rich in cells of the reticuloendothelial system were not examined.

The sponsor has concluded from this study that the "lipid composition of AmBisome with or without amphotericin B confers specificity for the site of fungal infections." However, using a different methodology other studies have shown the presence of amphotericin B in kidney and other tissues obtained from uninfected mice treated with either AmBisome or amphotericin B (Table 43, reproduced from Gondal *et al.*, 1989, Antimicrob Agents Chemother 33: 1544). In the studies sponsored by the company, it would have been worthwhile to examine the distribution of fluorescence labeled drug in tissues obtained from uninfected mice treated with labeled liposomes with and without drug *in vivo* to compare the relative intensity and localization of staining in different tissues in infected vs. uninfected mice. It would also have been worthwhile to include free amphotericin B as a control to determine whether the liposomal preparations actually do promote better localization of the drug in infected tissues.

TABLE 43 Organ concentration of AMB after i.v. injection of AMB and lip-AMB into normal mice[a].

Time postinjection (h)	AMB conc (μg/g)																	
	Liver			Spleen			Kidney			Brain			Heart			Lung		
	AMB	Lip-AMB		AMB	Lip-AMB		AMB	Lip-AMB		AMB	Lip-AMB		AMB	Lip-AMB		AMB	Lip-AMB	
		1	5		1	5		1	5		1	5		1	5		1	5
0.08	2.7	2.8	6.5	3.0	3.0	6.0	1.8	1.5	3.5	ND[b]	ND	0.03	0.02	ND	0.9	1.8	3.3	18.5
0.25	2.7	3.6	8.5	3.4	5.7	9.8	1.2	1.2	3.6	ND	ND	0.03	ND	ND	1.0	2.7	7.6	13.6
1	2.2	3.8	13.3	4.2	8.0	16.6	0.68	0.90	2.7	ND	ND	0.45	ND	ND	1.2	3.0	3.6	13.0
2	2.0	4.4	14.5	5.3	8.4	22.7	0.45	0.87	1.8	ND	ND	0.61	ND	ND	0.44	2.5	3.3	13.4
4	1.5	5.4	20.2	1.96	8.3	11.2	0.40	0.77	1.2	ND	ND	0.32	ND	ND	0.03	2.6	2.5	9.4
12	1.0	5.4	25.7	0.12	5.6	9.0	0.03	0.50	0.8	ND	ND	0.04	ND	ND	0.03	0.09	1.2	8.4
24	0.9	4.4	29.7	ND	4.2	6.6	0.03	0.50	0.7	ND	ND	0.01	ND	ND	0.02	0.06	0.09	3.4
48	ND	3.8	21.2	ND	2.1	5.3	ND	0.54	0.6	ND	ND	ND	ND	ND	ND	ND	ND	3.1

[a] Values are means for three mice. The AMB dose was 1 mg/kg, and the lip-AMB dose was either 1 or 5 mg/kg.
[b] ND, Not detectable.

Localization and integrity of the liposomal preparation *in vitro* using murine macrophages infected with *C. glabrata*

The localization and integrity of the liposomal preparation was examined *in vitro* using the technique of resonance energy transfer (report no. 232-93097). Murine peritoneal macrophages were infected with *C. glabrata in vitro* for 3 hours and the cells were washed to remove unphagocytosed yeast. Infected and uninfected macrophages were stained with dual fluorescent labeled AmBisome or empty liposomes [7-nitrobenz-2-oxa-1, 3-diazol-4-yl phosphatidylethanolamine (NMD-PE) and N-lissamine rhodamine B sulfonyl phosphatidylethanolamine (L-Rho-PE)] for a period of 1.5 or 5 hours. The distribution and integrity of the labeled AmBisome or empty liposomes was followed by fluorescence microscopic examination. The viability of the yeast cells within the macrophages was determined by staining with methylene blue. Results in Table 44 show a decrease in viability of the yeast cells after exposure to fluorescein labeled AmBisome. Although it has been stated that macrophages treated with AmBisome retained normal morphology unlike the cells exposed to buffer or the drug free liposomes, the effect on the viability of macrophages was not shown. The activity of AmBisome was not compared with the free form of the drug i.e., amphotericin B. Fluorescence microscopic examination of the infected and uninfected macrophages at 1.5 hours of exposure to labeled AmBisome or free liposomes showed an orange-yellow fluorescence thereby indicating an intact liposomal preparation. At 5 hours post-exposure the fluorescence observed was yellow-green in color indicating that the integrity of the labeled AmBisome was lost. Overall, these results suggest that AmBisome, like empty liposomes, can enter the macrophages irrespective of the presence of infection, wherein the integrity of the liposomal preparation is lost. Amphotericin B is effective in reducing the viability of intracellular *C. glabrata.* However, the effect on the viability of macrophages is not clear.

Table 44

Percent Viability of Phagocytized *C. glabrata* after 5 Hours of Incubation in Murine Peritoneal Macrophages

Percent viability of yeast cell inoculum = 96%
Percent viability of yeast after 5 hour intracellular incubation = 83%

Amphotericin B (µg)	NBD-PE/ L-Rho-PE Liposomes without drug	NBD-PE/ L-Rho-PE AmBisome
25	-	51
40	91	29

Binding of fungi to labeled AmBisome *in vitro*

Binding of fluorescein labeled (sulforhodamine) AmBisome or empty liposomes to *C. albicans* was investigated *in vitro* (report no. 232-93092). Incubation of *C. albicans* for 24 hours with drug free labeled liposomes resulted in surface labeling; whereas exposure to labeled AmBisome resulted in cytoplasmic staining of yeast or hyphae cells. These results show that presence of amphotericin B in the liposomal preparation disrupts the integrity of the fungi leading to penetration of the dye in to the cell. A similar observation was made when *C. albicans* was exposed to unlabeled AmBisome + free dye. Free dye alone did not result in cytoplasmic staining of the fungal organisms.

In another study, the integrity of the liposomes after exposure to *C. albicans* or *C. glabrata* was investigated by resonance energy transfer (report no. 232-93093). *C. albicans* and *C. glabrata* organisms were exposed to AmBisome or empty liposomes dual labeled with fluorescein dyes [7-nitrobenz-2-oxa-1, 3-diazol-4-yl phosphatidylethanolamine (NMD-PE) and N-lissamine rhodamine B sulfonyl phosphatidyl-ethanolamine (L-Rho-PE)] and followed by fluorescence microscopic examination at different time intervals for 21 to 24 hours. Results show that the integrity of the dual labeled AmBisome but not drug free liposomes is lost upon exposure to fungi *in vitro*. It was also shown that labeled AmBisome is effective in disrupting the integrity of fungal cells leading to reduced viability. Such an effect was initially observed 5.5 hours post-exposure of *C. albicans* and *C. glabrata* to AmBisome but increased over the period of observation. Labeled empty liposomes without the drug did not alter the viability or produce cytoplasmic staining of the fungi.

In another experiment, localization of liposomal lipid was investigated *in vitro* against *C. albicans*, *C. glabrata* and *A. fumigatus* using AmBisome or drug free liposomes labeled with L-Rho-PE (report no. 232-93094). Fluorescence examination showed drug free liposomes attach to the surface of fungal cells but were not effective in penetrating the fungal cells during the 22 to 28 hours of observation; whereas AmBisome bound to the surface and was effective in penetrating the fungal cells within 2 to 6 hours of incubation. The viability of the fungi coupled with the surface labeling suggest that lipid component of the AmBisome may penetrate into the cytoplasm of the fungi once the integrity of the cell wall is lost.

Electron microscopic examination (subcellular localization) of *C. albicans, C. glabrata* and *A. fumigatus* after exposure to AmBisome or drug free liposomes was performed either by freeze-fracture or by incorporation of nanogold particles into the liposomal preparation (report no. 232-93107). Freeze fracture studies show that AmBisome and drug free liposomes attach to the cell wall of *C. glabrata*. Intracellular localization could not be studied by this method. However, electron microscopic examination of *C. albicans, C. glabrata* and *A. fumigatus* after exposure to gold labeled AmBisome or drug free liposomes confirmed the findings observed using fluorescein labeled formulations. AmBisome penetrates through the cell wall and cell membrane into the cytoplasm. Drug free liposomes remained attached to the cell wall during the 24 hour period of observation.

Comments:

The sponsor has studied the stability of AmBisome in rat plasma, the effect of AmBisome on red blood cells *in vitro*, the ability of AmBisome to kill the fungal infection (*C. albicans*) in the kidneys of infected mice, the localization of the drug in the infected and uninfected murine macrophages *in vitro*, the localization of the liposome at the subcellular level and integrity of the liposomal preparation after entry into the fungus.

Overall, these studies show that AmBisome has the ability to enter the fungal cytoplasm either extracellularly or intracellularly (within the macrophages) and to exhibit activity against the fungal infection. However, these studies do not show that AmBisome preferentially localizes in the infected sites. In fact, the preponderance of data in the open literature show that liposomal preparations have an affinity for cells of the reticuloendothelial system irrespective of the infected state. Such a preferential affinity increases the interaction of ingested fungal organisms with the drug.

Drug Resistance

Resistance to amphotericin B is considered rare and the relevance of drug resistance to clinical outcome has not been established. Reports of its occurrence in nature are often conflicting, difficult to interpret and are of questionable clinical relevance. The lack of standardization in methods for determination of MICs has also contributed to the uncertainty regarding the exact incidence of resistance. Mutants with decreased susceptibility to amphotericin B have been isolated after passage of fungal species in media containing the drug and from patients receiving drug therapy. Many, but not all of these mutants, have a decreased concentration of ergosterol in their cell membranes or ergosterol precursors with lower affinity for amphotericin B. Several investigators have reported that fungi with higher mean minimum inhibitory concentrations (> 0.8 ug/ml) were more often the cause of fatal infections. Investigators have also isolated *Candida* with reduced susceptibility to amphotericin B from patients who have not received polyene therapy and from patients who show no evidence of infection by a drug resistant organism. While a trend to higher MICs is seen following polyene therapy, the more resistant organisms may be less virulent. The success of drug therapy, therefore, can not be readily predicted from MIC values, and failure of the drug can not always be attributed to reduced susceptibility of infecting strains, especially in the face of multiple immune defects existing in a typical patient. Nevertheless, a brief statement should be included in the label indicating a potential for the development of resistance.

The mechanism by which resistance develops is not known. There is some evidence to suggest that a reduction in the ergosterol content of the fungal cell membrane may contribute to decreased susceptibility to amphotericin B. It is also of note that treatment with azoles such as fluconazole (which decreases the synthesis of sterols) can decrease susceptibility to amphotericin B, however, amphotericin B-resistant mutants of *C. albicans* with a high ergosterol content have also been isolated (Hamilton-Miller, J.M.T. 1972, J Gen Microbiol 73: 201). In addition, decreased production

of hydrogen peroxide by phagocytic cells and an increase in the concentration/activity of the catalase enzyme have also been implicated as causative factors for induction of resistance (due to a decrease in oxidative cell damage). The concentration of amphotericin B employed may have a role in resistance development depending on the component affected.

Catalase enzyme has been shown to decrease the antifungal activity of amphotericin B (Sokol-Anderson *et al.*, 1986 J Inf Dis 154: 76; Sokol-Anderson *et al.*, 1988, Antimicrob Agents Chemother 32: 702). Exposure of an amphotericin B sensitive strain of *C. albicans* to catalase or catalase + superoxide dismutase (SOD) decreased the antifungal activity of amphotericin B *in vitro* under hypoxic (high nitrogen) conditions. SOD alone was not effective in modulating such an effect. It is also of note that these *in vitro* experiments were conducted by adding the enzymes 10 minutes prior to the addition of amphotericin B. The growth of *C. albicans* was also inhibited in medium containing n-alkane (medium which was earlier shown to increase the production of catalase) as compared to glucose. This increase in catalase activity was associated with increased resistance to amphotericin B as measured by a decrease in potassium or protein leakage, and an increase in the viability of the fungal cells as compared to the untreated cultures. Such an inactivation is not a direct action on the activity of the drug but is mediated through increased activity of the enzyme catalase. However, the correlation of the *in vitro* finding to the activity *in vivo* has not been investigated.

Drug Interactions

Drug interactions of amphotericin B with other antifungals and AZT have been reported. The data are somewhat anomalous. Some studies have demonstrated an antagonistic interaction between amphotericin B deoxycholate and the imidazole derivatives, miconazole and ketoconazole. As these compounds inhibit the synthesis of ergosterol, the antagonism is probably due to a reduction in the binding sites for amphotericin B in the fungal cell wall. However, other studies have shown that polyene-imidazole interactions can be synergistic, additive or indifferent. The discrepancy in these results probably reflects differences in the isolates (genera, species, strains) used in various studies, as well as variability in test methods. In general, the preponderance of evidence indicates antagonism between polyenes and imidazoles, consequently, this combination should be avoided when possible.

In vitro studies have demonstrated synergistic or indifferent interactions between rifampin and amphotericin B. Studies *in vivo* are less clear.

The interaction between amphotericin B and AZT or other approved antiretrovirals has not been directly examined. The methyl ester of amphotericin B methyl (AME) and AZT were synergistic in inhibiting HIV antigen production and viral CPE in *in vitro* studies. It has also been reported that amphotericin B alone significantly reduced HIV infectivity *in vitro*.

Considerations for drug interactions between AmBisome and other drugs may be similar to those observed for amphotericin B. There is only one report available wherein the antifungal activity of initial treatment with AmBisome (initiated 6 hours after infection) for 5 days followed by prolonged treatment with fluconazole for 13 days was examined in leukopenic mice infected with *C. albicans*. Results in Table 17 (page 13) show that maintenance therapy with AmBisome + fluconazole did not alter the activity as compared to the group treated with AmBisome alone. However, the effect of initial treatment with fluconazole followed by treatment with AmBisome was not investigated.

THE LABEL PROPOSED BY THE SPONSOR

CLINICAL PHARMACOLOGY

Mechanism of Action

AmBisome's targeted mechanism of action is due to several factors which result in a fungicidal concentration of amphotericin B being delivered specifically to the fungal cell membrane. Amphotericin B is firmly anchored in the stable liposome bilayer. AmBisome remains as the intact liposome and stays in circulation at high concentrations for prolonged periods of time. This results in a wide distribution to tissues where infections occur, but with less toxicity to normal cells when compared to traditional amphotericin B. AmBisome then localizes in infected tissues and cells and interacts directly with the fungus to produce an enhanced fungicidal effect. The liposomes attach directly to produce an enhanced fungicidal effect. The liposomes attach directly to the fungal cell wall where local interaction with the cell surface occurs. This interaction disrupts the liposome and amphotericin B is delivered into the fungal cytoplasm.

PHARMACOKINETICS

MICROBIOLOGY

AmBisome has shown *in vitro* activity equal to amphotericin B (within one dilution) against the following organisms:

Aspergillus species: *fumigatus, flavus*

Candida species: *albicans, glabrata, guillermondi, krusei, lusitaniae, parapsilosis, tropicalis*

Cryptococcus neoformans

Fusarium species

Blastomyces dermatitidis

However, standardized techniques for susceptibility testing of antifungal agents have not been established and results of such studies do not necessarily correlate with clinical outcome.

AmBisome is active in animal models against *Aspergillus fumigatus*, *Candida albicans*, *Candida krusei*, *Candida lusitaniae*, *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Trichosporon beigelii*, *Leishmania donovani* and *Leishmania infantum*. The administration of AmBisome in these animal models demonstrated prolonged survival of infected animals and clearance of microorganisms from target organs. The administration of AmBisome in these animal models demonstrated prolonged survival of infected animals and clearance of microorganisms from target organs.

CONCLUSIONS:

AmBisome, a liposomal preparation of amphotericin B, is an approved antifungal agent in the United Kingdom, some parts of Europe and Mexico. Amphotericin B is a well known antifungal agent. In this submission the sponsor seeks approval of AmBisome for treatment and/or prophylaxis of a wide variety of fungal infections and a protozoan parasite, *Leishmania*.

Antifungal activity

The antifungal activity of free amphotericin B is well known. The activity of the new liposomal formulation was investigated *in vitro* and *in vivo* and compared with that of the free drug. The *in vitro* studies indicate that the minimal inhibitory concentrations for AmBisome against different fungal species [*Candida albicans*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, C. *tropicalis*, *Aspergillus fumigatus*, *A. flavus*, *Cryptococcus neoformans*, *Fusarium* sp. (species not specified), and *Blastomyces dermatitidis*] are comparable to those observed using the free form of the drug. The sponsor used an *in vitro* model of murine peritoneal macrophages infected with *Candida* (*Torulopsis*) *glabrata* to study the localization of labeled AmBisome as compared to empty liposomes. The results show that AmBisome containing amphotericin B at 25 and 40 ug inhibited the viability of yeast cells to 51% and 29% respectively. Viability was determined by staining with methylene blue. There was no comparison made with the free drug and traditional MIC values were not determined. There were no studies done to demonstrate the activity of AmBisome against *C. guillermondi*. The time kinetics studies show that the killing effect of amphotericin B compared to AmBisome against *C. albicans* is more rapid and sensitive (within a period of 6 hours). However, at 24 hours, the activity of the two formulations was similar. Such an observation is not unexpected since the active drug ingredient i.e., amphotericin B is enclosed within the liposomes and may need additional time to reach sufficient concentrations to exhibit an antifungal effect.

The *in vivo* activity of AmBisome was tested against a wide range of fungal species as a prophylactic and/or therapeutic agent. Against *C. albicans*, AmBisome, when administered as a single prophylactic dose, was shown to be effective in reducing (not clearing) infection in the kidneys of

immunocompetent and immunosuppressed mice. Other organs were not tested. The activity of the drug was comparable to that of amphotericin B.

The therapeutic activity of AmBisome was demonstrated in immunocompetent and immunosuppressed mice infected with *C. albicans*. The activity of the drug depends on the severity of infection at the time treatment is initiated. A comparison of the activity of AmBisome with amphotericin B indicates that AmBisome can be administered at higher doses and, therefore, is more effective in improving the survival and reducing the fungal burden. However, a comparison of the same dose of the two formulations indicates free drug to be superior to / or comparable with the liposomal preparation. Depending on the strain of mice used, immune status of the host, severity of infection and other experimental conditions, free amphotericin B could not be administered at a $\geq$ 1.5 mg/kg dose. It is also of note that a decrease in fungal burden was measured for up to a period of 2 weeks after discontinuation of treatment. At this time a significant concentration of drug could be measured in tissues obtained from immunosuppressed infected mice. Therefore, the possibility of a relapse of infection after drug is completely eliminated from the tissues could not be ruled out.

Studies in immunosuppressed mice infected with other species of *Candida* which include *C. krusei* or *C. lusitaniae* suggest that the activity of AmBisome is comparable to that of amphotericin B in improving the survival rate or reducing the fungal burden. The mice employed in these studies were immunosuppressed but the immunosuppression was transient. Therefore, the contribution of immune cell function in the recovery from this infection could not be ruled out.

The activity of AmBisome against *A. fumigatus* was measured in immunosuppressed animals. When administered as a prophylactic agent to immunosuppressed mice, AmBisome like amphotericin B, was effective in improving the survival and reducing the fungal load in the lungs from infected mice. It is of note that the prophylactic activity of the drug varied with the challenge dose of organisms. The period of observation was 9 days after administration of the single dose of the drug and 3 doses of cortisone acetate. Therefore, the effect of residual drug in the tissue or the recovery from immunosuppression on the observed antifungal activity could not be determined. Therapeutic studies were conducted in rabbits which were immunosuppressed for a longer period of time and authors have stated that agranulocytopenia (< 100 cells/ul) was maintained from day 5 on. AmBisome was effective in reducing fungal burden and improving the survival of these infected animals.

AmBisome improves the percent survival and reduces fungal burden in immunocompetent mice infected with *Cr. neoformans*, or the dimorphic fungi *B. dermatitidis* and *P. brasiliensis*. Activity against *H. capsulatum* or *T. beigelii* was measured in athymic or immunosuppressed mice, respectively. Studies show AmBisome to be marginally active in improving the survival rate and/or reducing the fungal burden in these infection models. The activity of AmBisome in mice infected with *C. immitis* was defined in a single study in immunocompetent animals using lung weight as the surrogate marker. Treatment with AmBisome reduced the lung weight. Fungal burden was not measured. Results of the survival data were not included in the published report.

Overall, the activity of AmBisome against various fungi was shown to be comparable to amphotericin B based on the maximal tolerated doses of the 2 formulations. The dose of AmBisome required for improved survival or to reduce fungal burden varied with the fungal species and may also depend on the extent of infection at the time treatment is initiated. Relapse of infection due to the presence of residual organisms was not measured in most studies. It should also be noted that immunosuppressed animals were not used to establish fungal infection in the majority of the studies submitted. Immunosuppression would mimic more closely the situation observed in patients receiving antineoplastic therapy or bone marrow transplantation. A comparison of the activity of AmBisome with other liposomal preparations of amphotericin B (AMPHOTEC or ABELCET) was not performed.

These studies show that AmBisome is less active than free amphotericin B on an equivalent dosage basis. However higher doses can be administered as a liposomal preparation leading to improved and prolonged activity of the drug (with reduced toxicity).

Anti-*Leishmania* activity

The activity of AmBisome was measured *in vitro* against the amastigote stage of *L. donovani*. Results show a decrease in the percentage of infected murine macrophages. However, the actual parasite count and the growth of the parasite over a period of time was not provided. The drug exposure time was also not specified. AmBisome was stated to have no activity against the promastigote stage of the parasite.

Studies *in vivo* show that AmBisome exhibits activity against *L. donovani* and *L. infantum* in immunocompetent animals. The activity against *L. donovani* was measured in mice, hamsters and squirrel monkeys. Activity against *L. infantum* was measured in mice and naturally infected dogs. The studies show that treatment with AmBisome can decrease the parasite load in liver and to a lesser extent in spleen.

Mechanism of Action

Amphotericin B is a polyene antibiotic with high affinity for the sterol component of the cell membrane. The binding of this drug with the sterol alters the membrane permeability leading to release of intracellular components into the extracellular space which results in cell death. Amphotericin B binds to not only fungal cells but also to mammalian cells thus causing severe toxicity. Nevertheless, the affinity of binding to ergosterol is higher than the affinity for cholesterol.

In addition to alterations in cell membrane permeability, amphotericin B also has a role in oxidative cell damage which is independent of the effect of drug on membrane permeability or the nature of sterol. This can cause cell death directly by auto-oxidation. On the contrary, amphotericin B can increase the concentration/activity of catalase enzyme which can inhibit oxidative cell damage.

The mechanism by which amphotericin B exhibits activity against the *Leishmania* parasite was not investigated. However, studies have shown that the major sterol content of the amastigote and/or promastigote stage of *L. tropica*, *L. donovani* and *L. mexicana* is ergosterol suggesting that the mechanism of action is similar to the anti-fungal effects. It is of note that amphotericin B does not exhibit activity *in vitro* against the promastigote stage of the parasite even though the sterol content is similar. The reasons for this differential effect is unclear.

Liposomes can serve as a means for delivering drugs preferentially to cells of the reticuloendothelial system. Some of the drugs such as amphotericin B and nystatin are toxic when administered in free form but have been shown to exhibit reduced toxicity without alteration in their activity when administered as a liposomal preparation.

AmBisome is a liposomal preparation containing amphotericin B complexed with cholesterol, hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylglycerol (DSPG). Amphotericin B is bound to the lipid component by noncovalent bonds. However, it is not clear exactly how these bonds break. The complex could be broken by either disequilibrium in the constituents of the liposomal preparation or by the action of the hydrolytic enzymes (lipases/phospholipases). These hydrolytic enzymes are active at acid pH and distribution within the body appears to be ubiquitous (i.e. present within the lysosomes, peroxisomes, plasma etc.). The fate of the different components of the liposomal preparation after dissociation is also not clear.

Some of the studies submitted by the sponsor in support of the stability of the liposomal preparation and the specificity of the drug delivery system are as follows:

The sponsor has studied the stability of AmBisome in rat plasma, the effect of AmBisome on red blood cells *in vitro*, the ability of AmBisome to kill the fungal infection (*C. albicans*) in the kidney of infected mice, the localization of the drug in the infected and uninfected murine macrophages *in vitro*, the localization of the liposome at the subcellular level and integrity of the liposomal preparation after entry into the fungus. It is of note that amphotericin B exhibits stable binding during the period of incubation, but the *in vitro* conditions employed reflect a static environment. These conditions may be very different from the *in vivo* conditions wherein the equilibrium will be more dynamic. In addition, the action of lipases is optimal at acid pH (pH 5), but the *in vitro* experiments were conducted at a neutral pH. Also, hydrolytic enzymes are known to be relatively unstable. The stability of the phospholipases (present in plasma) in the *in vitro* experiments is questionable.

Overall, these studies show that AmBisome has the ability to enter the fungal cytoplasm either extracellularly or intracellularly (within the macrophages) and to exhibit activity against the fungal infection. However, these studies do not show that AmBisome preferentially localizes in the infected sites. In fact, the preponderance of data in the open literature show that liposomal preparations have an affinity for cells of the reticuloendothelial system irrespective of the infected state. Such a preferential affinity increases the interaction of integrated fungal organisms with the drug. However, the fate of such a foreign body may vary in activated vs. nonactivated phagocytic cells.

Drug Resistance

Mutants with decreased susceptibility to amphotericin B have been isolated from several fungal species after serial passage in culture media containing the drug, and from some patients receiving prolonged therapy. However, the relevance of drug resistance to clinical outcome has not been established.

The mechanism by which resistance develops is not known. There is some evidence to suggest that a reduction in the ergosterol content of the fungal cell membrane may contribute to decreased susceptibility to amphotericin B. It is also of note that treatment with azoles such as fluconazole (which decreases the synthesis of ergosterol) can decrease susceptibility to amphotericin B. In addition, decreased production of hydrogen peroxide and increases in the concentration/activity of the catalase enzyme have also been implicated as causative factors for induction of resistance (due to a decrease in oxidative cell damage). The concentration of amphotericin B may also have a role in resistance development depending upon the component affected.

Drug Interactions

Interactions between amphotericin B and other antifungal agents and AZT have been reported. Due to the known effects of the azole antifungals on ergosterol synthesis concurrent or sequential imidazole-polyene therapy is not recommended. The interaction between amphotericin B and AZT has not been directly examined, but a strong synergistic effect of amphotericin B methyl ester on AZT activity *in vitro* has been reported.

Based on the studies reviewed, the following changes in the label are recommended (some of the proposed changes to the sponsor's version of the label are struck out and the recommended changes are underlined):

FDA's Version of the Label (Microbiology Section)

(to be moved to the end of Microbiology section)

MICROBIOLOGY

Mechanism of Action

Amphotericin B, the active ingredient of AmBisome, acts by binding to the sterol component of a cell membrane leading to alterations in cell permeability and cell death. Amphotericin B has a higher affinity for the fungal cell membrane but can also bind to mammalian cells leading to cytotoxicity.

Activity *in vitro* and *in vivo*

AmBisome has shown *in vitro* activity comparable to amphotericin B against the following organisms: *Aspergillus* sp. (*A. fumigatus*, *A. flavus*), *Candida* sp. (*C. albicans*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*), *Cryptococcus neoformans*, and *Blastomyces dermatitidis*. However, standardized techniques for susceptibility testing of antifungal agents have not been established and results of such studies do not necessarily correlate with clinical outcome.

AmBisome is active in animal models against *Aspergillus fumigatus*, *Candida albicans*, *Candida krusei*, *Candida lusitaniae*, *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Leishmania donovani* and *Leishmania infantum*. The administration of AmBisome in these animal models demonstrated prolonged survival of infected animals, and / or reduction of microorganisms from target organs or lung weight.

Drug Resistance

Mutants with decreased susceptibility to amphotericin B have been isolated from several fungal species after serial passage in culture media containing the drug, and from some patients receiving prolonged therapy. Drug combination studies *in vitro* and *in vivo* suggest that imidazoles may induce resistance to amphotericin B. However, the clinical relevance of drug resistance has not been established.

RECOMMENDATIONS:

This NDA is approvable with respect to microbiology pending an acceptable version of the label. If additional (post-marketing) clinical trials are planned the sponsor should consider the following:

1. Please evaluate the potential for development of resistance using samples collected from patients before and after treatment with AmBisome.

/S/

Shukal Bala
Microbiologist, HFD-590

CONCURRENCES:

HFD-590/ Deputy Dir. /S/ Signature 10/30/97 Date
HFD-590/Micro TL Signature 8/4/97 Date /S/ 8/4/97
CC:
HFD-590/Original NDA 50-740
HFD-590/Division File
HFD-590/Div Dir Reading File
HFD-590/Pre-Clin Dep
HFD-590/MO
HFD-590/Pharm
HFD-590/Chem
HFD-590/Micro TL
HFD-590/Review Micro
HFD-590/CSO/FrankE

APPEARS THIS WAY
ON ORIGINAL

CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER: 050740

CLINICAL PHARMACOLOGY AND BIOPHARMACEUTICS REVIEW(S)

Clinical Pharmacology/Biopharmaceutics Review

NDA: 50,740

Submission Dates: 11/12/96, 1/21/97, 4/17/97, 4/25/97

Generic Name, Strength and Formulation: Liposomal Amphotericin B 50 mg lyophilized powder for injection

Brand Name: AmBisome®

Date Assigned: 11/15/96

Applicant: Fujisawa USA Inc

Final Review: 12/10/97

Submission Code: 3P

Reviewer: Kofi A. Kumi, Ph.D.

This is a synopsis of the review for NDA 50,740. The individual data are on file in the Division of Pharmaceutical Evaluation III.

SYNOPSIS:

The applicant submitted a new drug application for the use of Liposomal Amphotericin B (AmBisome®) in empiric therapy, treatment of systemic and deep mycoses, cryptoccosis, treatment of fungal infections in patients who are refractory to amphotericin B deoxycholate, treatment of visceral leishmaniasis and for prophylaxis against systemic fungal infections. There are two other lipid based amphotericin B products (Abelcet® and Amphotec®) that are currently marketed for 2nd line therapy in patients who are refractory to conventional amphotericin B.

The applicant submitted one pivotal and 2 supportive pharmacokinetic studies and 2 case reports describing the disposition of amphotericin B (AmB) after administration of AmBisome (LAmB). The pivotal study (92-0022) was a dose escalating trial that evaluated the safety and pharmacokinetics of AmB after administration of LAmB in adults receiving antineosplastic therapy or bone marrow with fungal infections. This study was sponsored by Fujisawa; the supportive studies were sponsored by NeXstar Inc. who holds the patent for AmBisome and has been marketing it in Europe and other countries. Fujisawa has licensed from NeXstar the rights to market AmBisome in the United States. A comparative pharmacokinetic study between AmBisome and amphotericin B deoxycholate was not conducted. However, comparative efficacy and safety data of amphotericin B after administration of AmBisome and amphotericin B deoxycholate (conventional) was submitted to the clinical section of the NDA.

The pivotal pharmacokinetics study was a sequential dose escalation, multidose study in cancer chemotherapy or bone marrow patients. The dosing regimen studied ranged from 1 to 7.5 mg/kg/day infused over 1 hour period. Total (amphotericin B bound and unbound to liposomes) amphotericin B concentrations were determined. A validated

high performance liquid chromatography method was used to determine total amphotericin B concentrations. The analytical method did not differentiate between amphotericin B bound or unbound liposomes; hence, total amphotericin B concentrations were measured.

A summary of the mean pharmacokinetic parameters of total amphotericin B after 1st and last dose (3-20 days) in serum after AmBisome administration to patients with cancer or bone marrow transplantation receiving doses ranging from 1 to 7.5 mg/kg/day in the pivotal pharmacokinetic study are provided in the table below. This was a sequential dose escalating study and the patients were febrile, neutropenic and eligible for empiric therapy.

Summary of Pharmacokinetic Parameters of Total Amphotericin B (Mean±SD) Following Infusions of AmBisome over 60 minutes

DOSE (MG/KG	DOSE DAY	N	CMAX (µG/ ML)	AUC24 (µG*H/ ML)	AUC∞ (µG*H/ ML)	MRT (H)	T1/2 (H)	Vβ (L/KG)	VSS (L/KG)	CL (ML/H/ KG)
1.0	1	8	7.3 ± 3.8	27 ±14	32 ±15	12.2 ±6.8	10.7 ±6.4	0.58 ±0.40	0.44 ±0.27	39 ±22
	Last	7	12.2 ±4.9	60 ±20	66 ±21	9.1 ±2.2	7.0 ±2.1	0.16 ±0.04	0.14 ±0.05	17 ±6
2.5	1	7	17.2 ± 7.1	65 ±33	71 ±36	8.0 ±1.0	8.1 ±2.3	0.69 ±0.85	0.40 ±0.37	51 ±44
	Last	7	31.4 ±17.8	197 ±183	213 ±196	8.5 ±1.8	6.3 ±2.0	0.18 ±0.13	0.16 ±0.09	22 ±15
5.0	1	12	57.6 ±21.0	269 ±96	294 ±102	8.2 ±2.0	6.4 ±2.1	0.22 ±0.17	0.16 ±0.10	21 ±14
	Last	9	83.0 ±35.2	555 ±311	621 ±371	9.8 ±2.5	6.8 ±2.1	0.11 ±0.08	0.10 ±0.07	11 ±6
7.5	1	6	83.7 ±43.0	476 ±371	534 ±429	9.5 ±3.2	8.5 ±3.9	0.26 ±0.15	0.18 ±0.10	25 ±22
	Last	4	62.4 ±17.7	382 ±148	417 ±155	9.5 ±1.5	6.9 ±0.9	0.20 ±0.07	0.17 ±0.05	20 ±7

Distribution: The mean±SD volume of distribution at steady state (Vss) for doses of 1 - 5 mg/kg/day range from 0.44±0.27 to 0.16±0.10 L/kg; volume of distribution decreases with an increase in dose suggesting that distribution into tissues may be saturable. Case studies submitted confirm preclinical studies that indicate AmBisome is preferentially distributed into the liver, spleen, bone marrow and other cells of the reticuloendothelial system. The protein binding of amphotericin B is 90%; however, the binding characteristics of AmB after administration of AmBisome was not studied.

Metabolism: The metabolic fate of amphotericin B after administration of AmBisome was not evaluated. The metabolic fate of amphotericin B after administration of both AmBisome and amphotericin B deoxycholate is not known.

Excretion: The excretion of amphotericin B after administration of AmBisome was not evaluated. However, there are reports that less than 1% of an administered dose is excreted in the urine unchanged.

The half-life reported was calculated based on sampling during a dosing interval (24 hours). This may be the relevant half life since the majority of the area under the concentration time curve is accounted for within the initial 24 hours after dosing. The applicant re-computed the terminal elimination half-life using all available serum concentrations (1-49 days) upon the reviewer's request. The re-computed mean half-life for the 2.5, 5, 7.5 mg/kg/day doses were 153±144, 105±52 and 100±24 hours, respectively. This indicates that there may be a "depot" of amphotericin B in the tissues which is released slowly into the systemic circulation. In the supportive studies, amphotericin B concentrations were detected 11 days after administration of AmBisome to patients who were also receiving doxorubicin.

Dose Proportionality: The AUC_{24} ,AUC_{∞} and Cmax after administration of AmBisome up to doses of 5 mg/kg/day were not proportional to dose. The pharmacokinetics of amphotericin B after administration of AmBisome is nonlinear such that there is an increase in concentration with an increase in dose up to 5 mg/kg/day.

Special Populations:

Renal Impairment: There was no evaluation of the effect of renal impairment on the pharmacokinetics of amphotericin B after administration of AmBisome; however, there are reports indicating that very little of amphotericin B is excreted in the urine after administration of amphotericin B deoxycholate (conventional amphotericin B).

Pediatrics: No formal pharmacokinetic study in children were conducted; however, the pivotal clinical studies included pediatric patients and there was no significant difference in the efficacy profile and adverse events from that reported for adults.

Gender and Ethnicity: No separate analysis of gender or ethnicity was conducted.

Drug Interactions: No pharmacokinetic drug interactions were conducted. However, statements regarding possible adverse events when AmBisome is administered with cyclosporin A, antineoplastic agents, corticosteroids, corticotropin, digitalis glycosides, flucytosine, azoles, Leukocyte transfusions, other nephrotoxic medications and skeletal muscle relexants were mentioned in the draft label. These statements are similar to that reported for conventional amphotericin B.

Concentration Effect Relationship (PK-PD): No concentration effect relationship evaluation was conducted after administration of AmBisome. The correlation between amphotericin B concentration and clinical outcome is not known.

The following table summarizes the pharmacokinetics of amphotericin after administration of AmBisome, Abelcet, Amphotec and Fungizone. This table was compiled based on information from the approved Abelcet®, Amphotec® labels and the data from study 92-0022 in the NDA for AmBisome. It must be noted that the method of data analysis is different for the different products (see footnotes for table).

Mean Pharmacokinetic Parameters of Approved Amphotericin B Formulations*

Pharmacokinetic Parameters	AmBisome 5 mg/kg/day (n=9)[ef]	Abelcet 5 mg/kg/day	Amphotec 5 mg/kg/day (n=51)[df]	Fungizone 0.6 mg/kg/day (n=5)[af]
Cmax (µg/mL)	83.0±35.2	1.7±0.8 (n=10)[b]	3.1	1.1±0.2
AUC24 (µg*h/mL)	555±311	14±7 (n=14)[b,c]	43	17.1±5.0
Cl (mL/h*kg)	11±6	436±188.5 (n=14)[b,c]	117.0	38±15
Vdβ (L/kg)	0.11±0.08 (0.10±0.07)	131±57.7 (n=8)[c]	(4.3)	5±2.8
Half Life (h)	6.8±2.1[g]	173.4±78 (n=8)[c]	28.6	91.1±40.9

[a] Data from Mucocutaneous Leishmaniasis patients. Infusion rate was 0.25 mg/kg/h

[b] Data across studies in patients with cytologically proven cancer being treated with chemotherapy or neutropenic patients with presumed or proven fungal infection. Infusion rate was 2.5 mg/kg/hr

[c] Data from mucocutaneous Leishmaniasis patients. Infusion rate was 4 mg/kg/h

[d] Predicted data obtained using population modeling in 51 bone marrow transplant patients. The modeling assumes amphotericin B pharmacokinetics after administration of Amphotec is best described by a 2-compartment model. Infusion rate = 1 mg/kg/h.

[e] Data obtained from adult cancer and bone marrow transplant patients who were febrile and neutropenic. Infusion rate was 5 mg/kg/h.

[f] Abelcet, Fungizone data based on whole blood, Amphotec based on plasma and AmBisome based on serum total concentrations of amphotericin B.

[g] Based on serum concentration data for a dosing interval of 24 hours

The pharmacokinetics of amphotericin B after administration of AmBisome appear to be different when compared to that observed after administration of Abelcet, Amphotec and conventional amphotericin B (Fungizone). However, it must be noted that the patient populations, method of sampling, analytical methods and data analysis were different which may influence the magnitude of the differences observed. Despite the differences in pharmacokinetics, all three liposomal formulations of amphotericin B have been reported to be safer (e.g. less nephrotoxicity) than conventional amphotericin B.

Supportive Studies: The pharmacokinetic parameters of amphotericin B after administration of AmBisome reported in the two supportive studies were similar to those noted in the pivotal pharmacokinetic study. The pharmacokinetics of AmB after intravenous administration of AmBisome in the cancer patients produced high serum concentrations and had lower volumes of distribution, suggesting the drug was sequestered within the circulating liposomes in the vascular compartment. Amphotericin B pharmacokinetics after administration of AmBisome tended to be nonlinear such that higher than expected increase in concentration was observed with an increase in dose. The mean half-life (based on serum concentrations data for 24 hours) ranged

However, a mean half-life based on day 11 trough concentrations ranged

from 2 to 30 days. The data suggested that steady state was attained by the end of the 5-day dosing cycle; generally, between days 3 to 4.

Case Studies: In a 52-year heart transplant patient who was administered a total of 50 mg amphotericin B as amphotericin B deoxycholate initially and then switched to AmBisome (total dose 50 mg) due to toxicities experienced with amphotericin B deoxycholate, the extent of exposure to amphotericin B over a 24 hour period was greater following AmBisome compared to conventional amphotericin B administration. The clearance of amphotericin B was lower after AmBisome administration when compared to the clearance after conventional amphotericin B. The pharmacokinetics of amphotericin B after administration of AmBisome is consistent with the observations made in the pivotal pharmacokinetic study (92-0022). In three different patients, amphotericin B concentrations following administration of AmBisome were determined in various organs. The greatest amphotericin B concentrations after administration of AmBisome were found in the liver and spleen, organs of the reticuloendothelial system (RES). This is consistent with the theory that these type of liposomes are taken up preferentially by the RES. The trend observed is consistent with observation in preclinical studies in animal species.

Comments:

Consideration should be given to the following:

1. Examining the metabolic fate of amphotericin B after administration of AmBisome. Conducting radiolabeled, mass balance study. Based on the results of in vitro and in vivo metabolism, it is recommended that relevant drug interaction studies be conducted. The renal excretion of amphotericin B after administration of AmBisome should also be evaluated.

2. Evaluating the pharmacokinetics of amphotericin B following administration of AmBisome and conventional amphotericin B (Fungizone) in a comparative study.

3. Developing a sensitive analytical method to differentiate amphotericin B bound and unbound to liposomes.

4. Developing an in vitro release test which may be used as a quality control tool for future minor changes in manufacturing process and/or formulations.

5. Evaluating the influence of gender, ethnicity and age on the pharmacokinetics of amphotericin B after administration of AmBisome, in future studies.

Recommendation:

The pharmacokinetic studies submitted under the Human Pharmacokinetics and Bioavailability Section of NDA 50,740 to fulfill sections 320 and 201.5 of 21 CFR provided an initial understanding of the pharmacokinetics of amphotericin B after administration of AmBisome and support a recommendation for approval of this NDA. The sponsor should provide a plan for addressing the above comments.

/S/

APPEARS THIS WAY
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