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In Vitro Renal Toxicity and In Vivo Therapeutic Efficacy in Experimental Murine Cryptococcosis of Amphotericin B (Fungizone) Associated with Intralipid

VÉRONIQUE JOLY,[1] ROBERT FARINOTTI,[2] LINE SAINT-JULIEN,[1] MONIQUE CHÉRON,[3] CLAUDE CARBON,[1] AND PATRICK YENI[1]*

Laboratoire d'Etude des Infections Expérimentales, Faculté X. Bichat, and Institut National de la Santé et de la Recherche Médicale Unité 13, Université Paris 7,[1] Laboratoire de Toxicologie, Service de Pharmacie Clinique, Hôpital Bichat,[2] and Laboratoire de Physique et Chimie Biomoléculaire, Centre National de la Recherche Scientifique, Unité 198, Université Pierre et Marie Curie,[3] Paris, France

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We compared the experimental toxicities and activities of deoxycholate amphotericin B (d-AmB) dissolved in glucose (Dd-AmB) or mixed with 20% Intralipid (ILd-AmB). In vitro, ILd-AmB was less toxic than Dd-AmB against renal tubular cells in primary culture. In vivo, the toxicities and activities of Dd-AmB and ILd-AmB were studied in DBA2 mice with cryptococcosis. The maximum tolerated dose of intravenously administered d-AmB, i.e., the dose that induced less than 15% mortality because of toxicity, was 1.7 to 2.5 times higher when it was administered as ILd-AmB than when it was administered as Dd-AmB. Both treatments given intravenously at the same dose were equivalent for improving the survival of mice and reducing CFU counts in infected tissue, but at maximum tolerated doses, ILd-AmB (2 mg/kg of body weight) was more effective than Dd-AmB (0.8 to 1.2 mg/kg). AmB concentrations in spleen, liver, lung, and kidney were measured by high-pressure liquid chromatography 4 and 24 h after a single injection of 1.2 mg of Dd-AmB per kg, 1.2 mg of ILd-AmB per kg, or 2 mg of ILd-AmB per kg. In a given organ, AmB levels were similar after administration of 1.2 mg of Dd-AmB or ILd-AmB per kg but were significantly higher after administration of 2 mg of ILd-AmB per kg. The lower level of toxicity of ILd-AmB might be explained by circular dichroism experiments, showing that ILd-AmB contained 10-fold less soluble oligomeric AmB, which is believed to be the toxic form of the drug, than Dd-AmB. We conclude that ILd-AmB is as efficient as Dd-AmB and is better tolerated than Dd-AmB in mice with experimental cryptococcosis. By allowing higher doses of AmB to be infused, Intralipid enhances AmB concentrations in infected sites, and thus the therapeutic activity of the drug.

Amphotericin B (AmB) remains the drug of choice for treating most systemic mycoses caused by opportunistic fungi (13, 30), but its use is restricted because of severe side effects, the most important being nephrotoxicity, which often limits the duration of the treatment (10, 36). Nephrotoxic side effects include reduction of the glomerular filtration rate, vasoconstriction (6, 34), and abnormalities of tubular functions (5, 6, 12).

Several groups of investigators have shown that incorporation of AmB into liposomes reduces its toxicity, allowing larger doses to be administered and thereby increasing its efficacy in both experimental (29, 32, 37) and clinical (27, 28, 35) studies. Some of these formulations are now available in clinical practice. They display significant antifungal activity, but are not devoid of drawbacks; they may exhibit some residual toxicity (8, 21) and are expensive. Intralipid is a lipidic emulsion commercially available for parenteral nutrition. Experimentally, a delivery system of AmB emulsified in 20% Intralipid (ILd-AmB) was shown to be less toxic and more effective in the treatment of experimental candidiasis than the commercial formulation of AmB for the intravenous route, i.e., deoxycholate AmB (d-AmB) dissolved in glucose (Dd-AmB) (22). It has been shown more recently, in neutropenic patients, that d-AmB directly emulsified in 20% Intralipid (ILd-AmB) is responsible for fever, rigors, and a decrease in creatinine clearance less often than Dd-AmB (31), whereas it remains effective in the treatment of systemic candidiasis (7).

Cryptococcus neoformans is an encapsulated yeastlike fungus responsible for meningoencephalitis. This severe disease is observed in patients with AIDS, occurring in 30% of cases in some countries (14). Although azoles represent an interesting alternative therapy to Dd-AmB, treatment failures have been reported in patients with severe forms of the disease (23), and Dd-AmB is often indicated at the initial phase of the treatment (9). Thus, a formulation of AmB that is less toxic than Dd-AmB and that does not have the drawbacks of liposomal AmB would be useful for improving the treatment of cryptococcosis. Since patients with AIDS differ from neutropenic patients hospitalized in intensive care units, and since *C. neoformans* infects the central nervous system, in contrast to *Candida albicans*, it appears that the results obtained in neutropenic patients with suspected or proven candidiasis could not be directly extrapolated to patients with AIDS and cryptococcal meningoencephalitis. Therefore, an evaluation of ILd-AmB in an experimental model of *C. neoformans* appeared to be important before considering clinical studies.

Here we report the experimental toxicity and activity of ILd-AmB. Toxicity was evaluated in vitro against renal tubular cells in primary culture and in vivo by determination of the maximum tolerated dose of the drug in mice infected with *C. neoformans*. Antifungal activity was assessed in vivo in the model of cryptococcosis in mice. The toxicity and efficacy of ILd-AmB were compared with those of Dd-AmB, the conventional form of the drug for intravenous administration.

* Corresponding author. Mailing address: Service de Médecine Interne, 10 ème étage, Hôpital Bichat, 46, rue Henri Huchard, 75018 Paris, France.

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MATERIALS AND METHODS

Materials. Parenteral deoxycholate AmB (Fungizone) was obtained from Squibb (Neuilly-sur-Seine, France). The 20% Intralipid was obtained from Kabi Pharmacia (Saint Quentin en Yvelines, France). Ethanolamine, insulin, transferrin, hydrocortisone, triiodothyronine, sodium selenite, Percoll, collagenase type I, and collagen type 1 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Radiolabeled tracer $K_2H^{32}PO_4$ was obtained from New England Nuclear (Boston, Mass.). Culture media were from Flow Laboratories (Irvine, United Kingdom).

Preparation of solutions. (i) Conventional AmB. Parenteral AmB (Fungizone) was dissolved in 5% glucose (Dd-AmB) at a concentration of 5 mg/ml and was further diluted in 5% glucose for in vivo studies or in phosphate-buffered saline (PBS; pH 7.4) for in vitro experiments.

(ii) ILd-AmB. Parenteral AmB was directly emulsified in 20% Intralipid (ILd-AmB) at a concentration of 5 mg/ml. The vial was vigorously vortexed for at least 2 min, until a homogeneous yellow formulation was obtained. The initial preparation was further diluted in 20% Intralipid, and the mixture was used immediately.

Determination of AmB binding to Intralipid by circular dichroism analysis. We used circular dichroism as described previously (20). ILd-AmB at an initial concentration of 5 mM was added to 20% Intralipid diluted 500-fold in PBS, so that the final concentration of AmB was 5 μM. At this concentration of d-AmB, the circular dichroism spectrum of free AmB consisted of an intense dichroic doublet centered at 340 nm. Since AmB bound to phospholipids does not exhibit this dichroic doublet, the intensity of the doublet allowed us to determine the amount of unbound drug present in each formulation.

Toxicity against renal tubular cells in primary culture. (i) Cell culture. Kidneys were excised from male New Zealand rabbits (weight, 800 to 1,000 g; Evic Ceba, Blanquefort, France) under anesthesia, and primary confluent monolayers were grown from proximal tubule fragments in serum-free medium as reported previously (16, 18). Serum-free medium, referred to as culture medium, consisted of a 1:1 (vol/vol) mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–21.5 mM HCO_3^-–1 mM sodium pyruvate–4 mM L-glutamine–10 ml of a 100× nonessential amino acid mixture per liter–50 IU of penicillin per ml–50 μg of streptomycin per ml–100 nM sodium selenite–35 mg of transferrin per liter–5 mg of insulin per liter–100 nM hydrocortisone–20 μM ethanolamine. The cultures became confluent in 5 to 6 days. All experiments described here were carried out on day 7.

(ii) Exposure of cells to Dd-AmB and ILd-AmB. The culture medium was changed on the day before the experiments. On day 7, cells grown to confluence were incubated with fresh culture medium containing the appropriate concentrations of Dd-AmB or ILd-AmB for 1 h.

(iii) P_i uptake. P_i uptake was chosen as the marker of early tubular toxicity, as described previously (16, 18). Dd-AmB or ILd-AmB was removed after 1 h of incubation by washing, and P_i uptake was measured as reported elsewhere (16, 18). Briefly, P_i uptake was determined at 37°C in a buffered uptake solution of the following composition (mmol per liter): 137 NaCl, 5.4 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, and 15 HEPES (pH 7.4). Choline chloride replaced sodium chloride in the sodium-free solution. Cells were washed twice with 0.5 ml of the appropriate ice-cold uptake solution per well and were then incubated for 10 min in the presence of $K_2H^{32}PO_4$ (0.5 μCi/ml) and 0.1 mM unlabeled KH_2PO_4. We verified previously that P_i uptake increases linearly with time up to 10 min. Uptake was stopped by washing the cells twice with 1 ml of ice-cold buffer (137 mM NaCl, 15 mM HEPES [pH 7.4]) per well. Cells were then solubilized in 0.5% Triton X-100 (250 μl per well), and aliquots were counted by liquid scintillation. The radioactive counts in each sample were normalized with respect to the protein content of each well, which was determined by the method of Bradford (3). Each experiment was run in triplicate.

Experimental cryptococcosis. (i) Model. Experimental cryptococcosis was induced in DBA/2 mice infected intravenously with 2×10^5 *C. neoformans*, an inoculum responsible for a delayed disease that is lethal in 15 days, as described previously (24). Yeast cells were counted before infection, and the precise number of viable cells injected was verified retrospectively by plating the inoculum on Sabouraud-chloramphenicol agar.

(ii) Survival study. In each experiment, treated groups (at least 10 mice per group) were compared with an untreated control group. The treatment was administered intravenously 9 days after infection, when animals exhibited the first symptoms of disease, and consisted of a single dose of Dd-AmB or ILd-AmB. Cages were monitored at least once daily to evaluate the viabilities of the animals.

We first studied rising doses of Dd-AmB or ILd-AmB in order to determine the maximum tolerated dose of each treatment, defined as the dose responsible for less than 15% mortality because of toxicity. Death related to d-AmB administration always occurred within 48 h following intravenous injection (data not shown). Since infected mice were treated 9 days following infection, mortality because of drug toxicity was not observed after day 11 postchallenge. In untreated controls, mortality because of infection did not occur before day 14 postchallenge. Therefore, the cause of mortality of infected mice treated with d-AmB could be assigned to drug toxicity or infection, depending on the time to death after infection.

Having determined the maximum tolerated dosage for each formulation, we compared the activities of the dosages in the treatment of cryptococcosis.

(iii) *C. neoformans* tissue counts. The number of viable fungi in the major organs was determined 3 and 10 days after treatment of DBA/2 mice infected with 2×10^5 *C. neoformans* and treated with Dd-AmB or ILd-AmB. Mice were treated on day 9 after infection and cryptococcal tissue counts were measured in animals that had received the maximum tolerated dose of Dd-AmB, the same dose of AmB given as ILd-AmB, and the maximum tolerated dose of ILd-AmB. In each experiment, values were compared with those obtained in mice infected with the same inoculum and left untreated. Mice were killed by cervical dislocation, and the CFU was counted after 10-fold dilutions of spleen, lung, kidney, and brain tissue homogenates were plated in duplicate on Sabouraud-chloramphenicol agar. Colonies were counted after 48 h of incubation at 37°C. The method was sensitive to ≥100 CFU/g.

Pharmacokinetic study. AmB concentrations in serum and organs were determined in a group of mice infected with *C. neoformans* and treated intravenously on day 9 with the same dose of Dd-AmB or ILd-AmB as that given to mice whose serum and organs were sampled for CFU determination. Mice were killed by cervical dislocation. A 0.5-ml blood sample was obtained via heart puncture, and the liver, spleen, left lung, and left kidney were sampled. Samples were kept at −20°C for

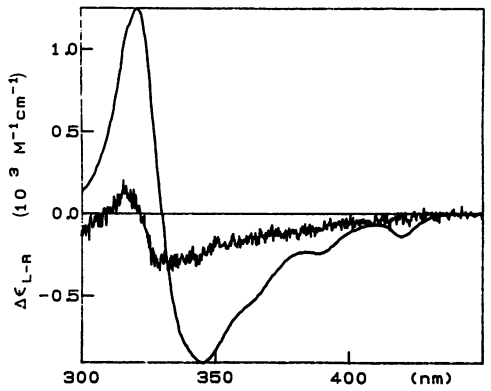

FIG. 1. Circular dichroism (Δ ε) of 5 μM AmB without (straight line) or with (irregular line) Intralipid.

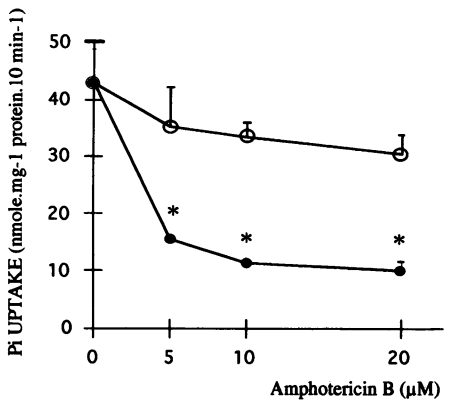

FIG. 2. Effect of Dd-AmB (●) and ILd-AmB (○) on Na^+-dependent uptake of P_i (0.1 mM) by renal tubular cells. Data are the means ± standard deviations of three determinations. *, significantly different from control ($P < 0.001$).

a duration that never exceeded 1 month. AmB was measured by liquid chromatography following extraction as described previously (26). Briefly, sera (100 μl) and tissue (50 to 150 mg) were extracted with 300 μl of methanol. The chromatographic system used a reverse-phase octadecylsilane column (Spherisorb ODS2, 5 μm; 4.6 by 150 mm). The mobile phase consisted of ammonium acetate buffer (1 mM; pH 6.6), tetrahydrofuran, and methanol (210:105:15; vol/vol/vol) with a flow rate of 0.8 ml/min. The UV detector wavelength was set at 410 nm. The method was linear from 0.1 to 2 μg/ml for serum and from 10 to 100 μg/g for tissues. The intraassay coefficients of variation were 8.2 and 4.0% for 0.1- and 1-μg/ml concentrations in serum, respectively, and 5.3 and 3.1% for 10- and 20-μg/g concentrations in lung samples spiked with AmB, respectively. Interassay coefficients of variation were 4.9% for 0.5-μg/ml serum samples and 6.2% for 10-μg/g spiked lung samples. The sensitivity of the method was 0.02 μg/ml and 0.5 μg/g for serum and tissue, respectively.

Statistical analysis. Survival curves were compared by the log rank test. Other data were analyzed statistically by using StatView software on a Macintosh LC computer. The Scheffe F-test was used for analysis of P_i uptake results and to compare the mean AmB concentrations measured in a given organ after the different therapeutic regimens, when allowed by the F value (analysis of variance). Fungal counts, expressed in $\log_{10}$ CFU per gram of organ, were compared between two groups by using the Mann-Whitney U-test, when the Kruskal-Wallis test, performed on more than two groups, was significant. For all of these tests, $P < 0.05$ was considered significant.

RESULTS

Measurement of the amount of free AmB in Dd-AmB and ILd-AmB preparations. For a 5-μM AmB concentration, the amount of free AmB was 10-fold lower in the ILd-AmB than in the Dd-AmB preparation, as shown by the measure of the dichroic doublet characteristic of the free drug (Fig. 1).

In vitro renal toxicity. Unlike Dd-AmB, which induced a significant decrease in P_i uptake for concentrations greater than 5 μg/ml, ILd-AmB was not toxic, even at 20 μg/ml, the highest concentration tested (Fig. 2).

In vivo toxicity and antifungal activity. (i) Survival curves. In the mouse experimental model used in the present study, drug toxicity and infection were two causes of death that could be differentiated according to the time to death following infection. Dd-AmB at 2 mg/kg of body weight and ILd-AmB at 2.5 mg/kg induced more than 60% immediate lethality. The maximum tolerated dose of ILd-AmB, defined as the dose responsible for less than 15% lethality, was 2 mg/kg in all experiments. The maximum tolerated dose of Dd-AmB differed slightly between the first and second sets of experiments (0.8 and 1.2 mg/kg, respectively) (Table 1). Thus, ILd-AmB was 1.7 to 2.5 times less toxic than Dd-AmB.

Figure 3 depicts a representative experiment in which the maximum tolerated dose of Dd-AmB was 0.8 mg/kg. When given at the same dosage (0.8 mg/kg), Dd-AmB and ILd-AmB increased the survival of mice compared with that of controls ($P < 0.01$), and the effect of treatment was indistinguishable. However, the maximum tolerated dose of ILd-AmB was more effective than the maximum tolerated dose of Dd-AmB ($P < 0.001$). A significant superiority of 2 mg of ILd-AmB per kg over 1.2 mg of Dd-AmB per kg ($P < 0.05$) was also observed in experiments in which the maximum tolerated dose of Dd-AmB was 1.2 mg/kg (data not shown).

(ii) CFU counts in tissues. CFU counts in all tissues except kidneys were obtained from two different experiments; kidneys were studied in the second experiment only. These results confirmed the results of the survival curves (Tables 2 and 3).

TABLE 1. Lethal toxicity of d-AmB administered intravenously in DBA/2 mice 9 days after infection with *C. neoformans*

Expt no.	No. of deaths/no. of treated mice (% mortality) after the following treatments (mg/kg):							
	Dd-AmB				ILd-AmB			
	0.8	1.2	1.6	2	0.8	1.2	2	2.5
1		3/21 (14)	7/8 (88)	6/6 (100)	ND[a]	0/16 (0)	1/17 (6)	13/19 (68)
2	0/9 (0)	7/14 (50)	ND	ND	0/10 (0)	ND	2/14 (14)	ND

[a] ND, not done.

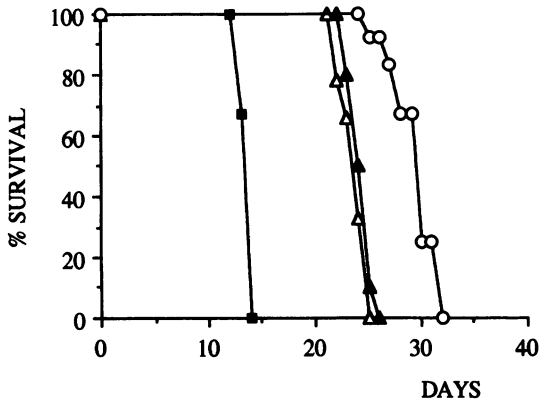

FIG. 3. Survival of DBA2 mice infected intravenously with 2×10^5 *C. neoformans* and treated 9 days after infection with a single injection of 0.8 mg of Dd-AmB per kg (△), 0.8 mg of ILd-AmB per kg (▲), 2 mg of ILd-AmB per kg (○), or PBS (■).

Three days after treatment, ILd-AmB at 2 mg/kg was more effective than the optimal dosage of Dd-AmB (1.2 mg/kg) at reducing CFU counts in the spleen ($P < 0.05$) and the brain ($P < 0.05$), but it was as efficient as 1.2 mg of Dd-AmB per kg at reducing the CFU counts in the lung and the kidney. Ten days after treatment, ILd-AmB at 2 mg/kg was more efficient than 1.2 mg of Dd-AmB per kg at reducing CFUs counts in the spleen ($P < 0.05$) and the lung ($P < 0.05$). CFU counts in the spleen, kidney, lung, and brain were similar in mice treated with 1.2 mg/kg of Dd-AmB or ILd-AmB at both 3 and 10 days after treatment.

Pharmacokinetic study. Results of the pharmacokinetic study were obtained from two different experiments and are presented in Fig. 4. AmB concentrations in tissues were not significantly different in mice treated with 1.2 mg of Dd-AmB or ILd-AmB per kg. AmB levels measured 4 h after treatment with the maximum tolerated dose of ILd-AmB (2 mg/kg) were higher than those obtained with 1.2 mg of Dd-AmB or ILd-AmB per kg in the spleen ($P < 0.01$), liver ($P < 0.05$), lung ($P < 0.001$), and kidney ($P < 0.01$). Concentrations measured in tissue 24 h after treatment remained significantly higher in mice treated with 2 mg of ILd-AmB per kg than in mice treated with 1.2 mg of Dd-AmB per kg for the spleen ($P < 0.01$), liver ($P < 0.001$), lung ($P < 0.001$), and kidney ($P < 0.001$) and than in mice treated with 1.2 mg of ILd-AmB per kg for the spleen ($P < 0.05$), liver ($P < 0.001$), lung ($P < 0.05$), and kidney ($P < 0.001$). Levels in serum at 4 and 24 h after injection were not significantly different between the three therapeutic groups (data not shown).

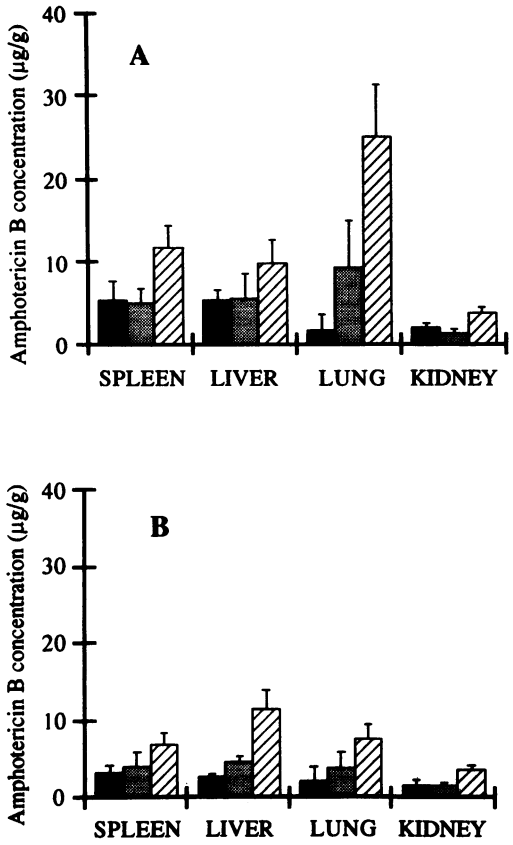

FIG. 4. (A) AmB concentrations in tissue measured in infected mice 4 h after a single intravenous injection of 1.2 mg of Dd-AmB per kg (■), 1.2 mg of ILd-AmB per kg (▦), or 2 mg of ILd-AmB per kg (▨). (B) AmB concentrations in tissue measured in infected mice 24 h after a single intravenous injection of 1.2 mg of Dd-AmB per kg (■), 1.2 mg of ILd-AmB per kg (▦), or 2 mg of ILd-AmB per kg (▨). Histograms and bars are means ± standard deviations obtained with results for from five to eight mice.

DISCUSSION

Many studies have shown the possibility of using liposomal AmB in the treatment of experimental systemic fungal and parasitic infections (29, 32, 37). Incorporation of AmB into liposomes usually leads to a formulation that is as effective as or less effective than conventional AmB given at the same dose. However, liposomes reduce the toxicity of the drug, and increasing the unitary dose and/or the duration of therapy

TABLE 2. Organism counts in organs measured 3 days after treatment of mice infected with 3×10^5 *C. neoformans*

Treatment	$\log_{10}$ CFU/g of organ (mean ± SD)			
	Spleen	Kidney	Lung	Brain
Control	6.13 ± 1.13 ($n^a = 6$)	7.62 ± 0.06 ($n = 3$)	7.68 ± 0.17 ($n = 4$)	7.31 ± 0.32 ($n = 7$)
Dd-AmB (1.2 mg/kg)	4.84 ± 0.63 ($n = 8$)	6.06 ± 0.37[b] ($n = 4$)	7.11 ± 0.23[b] ($n = 4$)	7.01 ± 0.11[b] ($n = 8$)
ILd-AmB (1.2 mg/kg)	5.10 ± 0.65 ($n = 8$)	6.39 ± 0.39[b] ($n = 4$)	6.91 ± 0.18[b] ($n = 4$)	7.00 ± 0.18[b] ($n = 8$)
ILd-AmB (2 mg/kg)	3.97 ± 0.60[c,d,e] ($n = 8$)	5.60 ± 0.37[b,d] ($n = 4$)	7.11 ± 0.05[b] ($n = 4$)	6.77 ± 0.24[b,d,e] ($n = 8$)

[a] n is the number of mice studied per group.
[b] Significantly different from control ($P < 0.05$).
[c] Significantly different from control ($P < 0.01$).
[d] Significantly different from ILd-AmB at 1.2 mg/kg ($P < 0.05$).
[e] Significantly different from Dd-AmB at 1.2 mg/kg ($P < 0.05$).

TABLE 3. Organism counts in organs measured 10 days after treatment of mice infected with 3×10^5 *C. neoformans*

Treatment	$\log_{10}$ CFU/g of organ (mean ± SD)			
	Spleen	Kidney	Lung	Brain
Dd-AmB (1.2 mg/kg)	5.03 ± 0.92 (n^a = 8)	5.77 ± 0.18 (n = 4)	6.67 ± 0.41 (n = 8)	6.47 ± 0.59 (n = 8)
ILd-AmB (1.2 mg/kg)	4.90 ± 0.80 (n = 8)	5.36 ± 0.44 (n = 4)	6.48 ± 0.67 (n = 8)	6.66 ± 0.67 (n = 8)
ILd-AmB (2 mg/kg)	3.81 ± 0.97[b,c] (n = 8)	5.14 ± 0.36 (n = 4)	5.60 ± 0.91[b] (n = 8)	5.60 ± 0.91 (n = 8)

[a] n is the number of mice studied per group.
[b] Significantly different from Dd-AmB at 1.2 mg/kg ($P < 0.05$).
[c] Significantly different from ILd-AmB at 1.2 mg/kg ($P < 0.05$).

without inducing severe side effects results in an overall beneficial effect of liposomal AmB. It has been shown that the liposomal structure of the vehicle is not required to enhance the therapeutic index of AmB (15). In an attempt to simplify the preparation of an AmB lipidic emulsion, we directly emulsified the commercial preparation of AmB, i.e., d-AmB, in 20% Intralipid, and the mixture was obtained after shaking by hand. We did not remove the deoxycholate and did not use additional methods in order to simulate conditions of easy preparation of ILd-AmB for patients. Galenic studies were not performed, and the type of interaction between AmB, deoxycholate, and Intralipid remains to be determined. It must be stressed that the effects of in vivo toxicity on mortality, in vivo efficacy, and pharmacokinetics were reproducible from one experiment to another, arguing for the reliability of the preparation when it is used immediately after reconstitution and infused as a bolus. The galenic modifications that may have occurred over time remain to be determined.

Pharmacokinetic studies of ILd-AmB and Dd-AmB were performed in infected mice in order to take into account the potential alterations in vascular permeability related to infection. The distribution of AmB in tissues was not modified by Intralipid, including the reticuloendothelial system organs, when evaluated 4 and 24 h after treatment. This differs from the results reported with small liposomes, which usually target AmB in the reticuloendothelial system (11, 33). The most striking differences between AmB concentrations measured after administration of 2 mg of ILd-AmB per kg and 1.2 mg of Dd-AmB per kg were observed in the lung, as described previously with some liposomal AmB formulations (39).

In vivo toxicity experiments were also performed in infected mice by using increasing doses of AmB given as the Dd-AmB or the ILd-AmB formulation, and their effects on early mortality were evaluated. Results showed that the maximum tolerated dose (inducing less than 15% mortality) of AmB was 1.7 to 2.5 times higher with ILd-AmB than with Dd-AmB. In vivo renal toxicity was not specifically evaluated. The lack of alteration of drug diffusion in the kidney by Intralipid does not preclude a protective effect against d-AmB nephrotoxicity; it has been shown that decreased toxicity could be observed with liposomes in the absence of a reduction in the concentration of AmB in renal tissue (26, 33). Recent clinical studies have confirmed the good renal tolerance to ILd-AmB in neutropenic patients receiving daily dosages in the range of 1 mg/kg (31). However, a significant increase in serum creatinine levels occurred in five of six human immunodeficiency virus-infected patients treated with a daily infusion of 1.5 mg of ILd-AmB per kg for cryptococcal meningitis (17).

Our in vitro results with renal tubular cells showed the reduced toxicity of ILd-AmB compared with that of Dd-AmB, and similar results have been obtained when the toxicity of liposomal AmB was evaluated against these target cells (16, 18). The level of protection afforded by Intralipid against d-AMB toxicity was higher in vitro than in vivo. In vivo, the toxicity of d-AmB was 1.7- to 2.5-fold lower in the presence of Intralipid, as shown by the comparison of the maximum sublethal doses in mice (0.8 or 1.2 versus 2 mg/kg), whereas in vitro, ILd-AmB remained less toxic than Dd-AmB, although ILd-AmB was tested at a 4-fold increased concentration. This apparent discrepancy might be explained by the binding of AmB to lipoproteins in serum (4, 38); if the avidity of AmB is higher for the lipid components in serum than for the lipid components in Intralipid, a progressive transfer of the drug may then occur from Intralipid to lipoproteins, back to the conventional situation of lipoprotein-bound AmB which is observed following intravenous infusion of Dd-AmB. Thus, our in vitro results may simply be explained by the lower free AmB fraction in the ILd-AmB formulation than in the Dd-AmB formulation.

The in vivo antifungal activity of AmB was not reduced by Intralipid, as shown by the similar efficacies of the same doses of Dd-AmB and ILd-AmB for improving the survival of mice or reducing CFU counts in infected tissues. The beneficial effect of the high dose (2 mg/kg) of ILd-AmB compared with that of the maximum tolerated dose (0.8 to 1.2 mg/kg) of Dd-AmB was associated with the higher concentrations of AmB measured in infected tissues. The decrease in the in vivo toxicity of d-AmB by Intralipid was therefore sufficient to improve the therapeutic index. This experimental model reflects rather well the systemic cryptococcosis observed in patients with AIDS. ILd-AmB at 2 mg/kg was more efficient than other regimens for reducing CFU counts in the brain on day 3 only, but it was always more efficient than other treatments in reducing CFU counts in the spleen. The reduction of the fungal burden in extracerebral foci of infection appeared to be important for delaying the evolution of disease. The small differences in the sizes of the CFU counts between control and treated mice may easily be explained by the fact that mice were treated with one injection of the drug; furthermore, this occurred late in the course of disease. However, comparison of the efficacies of a single dose of each formulation allows us to test the intrinsic in vivo activity of each formulation.

The spectroscopic investigation shed light on the possible mechanisms of protection afforded by Intralipid. Free (non-protein-bound) AmB is present in aqueous medium in three distinct physical states: soluble monomeric, soluble oligomeric, and insoluble aggregated. The concentration of the soluble oligomeric form of free AMB, which is thought to be the most toxic form (1, 2, 25), was reduced 10-fold in the presence of Intralipid, showing the existence of lipid-bound drug in the preparation. Jullien et al. (19) have shown that the amount of AmB remaining free in a preparation of liposomal AmB influences the in vitro cellular effects of liposomal AmB formulations. Since high concentrations of Intralipid prevented the observation of AmB circular dichroism because of

the strong scattering of the suspension, the lipid/AmB concentration ratio used in spectroscopic experiments had to be lower than that in the preparation of ILd-AmB used in toxicity and efficacy experiments. However, we have previously shown (16) with liposomes of pure phospholipid composition that increasing the lipid/AmB ratio favors the binding of AmB to lipids. We therefore anticipate that the amount of free AmB was significantly reduced in the final preparation used for in vitro and in vivo studies. Although the influence of deoxycholate on the interaction between lipids and AmB was not investigated, it is likely that it facilitated the solubility of unbound AmB in the aqueous phase, and thus its diffusion toward the lipid compartment.

In conclusion, ILd-AmB is a preparation of d-AmB which is more effective than Dd-AmB in the treatment of experimental systemic cryptococcosis when both drugs are given at the maximum tolerated dose. Intralipid did not specifically target AmB toward infected sites and did not alter the antifungal effect of a given dose, but it reduced the acute toxicity of the drug, permitting the administration of higher doses. The influence of Intralipid on the physical states of AmB in the ILd-AmB formulation remains to be evaluated. The efficacy of ILd-AmB in the treatment of cryptococcosis is of particular interest, since cryptococcosis is a severe infection in patients with AIDS for which an optimal therapeutic regimen remains to be determined. This is of particular importance in areas such as Central Africa, where the disease is highly frequent and no triazoles are available in case of Dd-AmB side effects, because of the costs of triazoles.

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REFERENCES

1. **Barcwicz, J., S. Christian, and I. Gruda.** 1992. Effects of aggregation state of amphotericin B on its toxicity to mice. Antimicrob. Agents Chemother. **36**:2310–2315.
2. **Bolard, J., P. Legrand, F. Heitz, and B. Cybulska.** 1991. One-sided action of amphotericin B on cholesterol-containing membranes is determined by its self-association in the medium. Biochemistry **30**:5707–5715.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brajtburg, J., S. Elberg, J. Bolard, G. S. Kobayashi, R. A. Levy, R. E. Ostlund, D. Schlessinger, and G. Medoff.** 1984. Interaction of plasma proteins and lipoproteins with amphotericin B. J. Infect. Dis. **149**:986–997.
5. **Burgess, J. L., and R. Birchall.** 1972. Nephrotoxicity of amphotericin B, with emphasis on changes in tubular function. Am. J. Med. **53**:77–84.
6. **Butler, W. T., G. J. Hill, and C. F. Szwed.** 1964. Amphotericin B renal toxicity in dog. J. Pharmacol. Exp. Ther. **143**:47–56.
7. **Caillot, D., O. Casasnovas, E. Solary, P. Chavanet, B. Bonnotte, G. Reny, F. Entezam, J. Lopez, A. Bonnin, and H. Guy.** 1993. Efficacy and tolerance of an amphotericin B lipid (Intralipid) emulsion in the treatment of candidaemia in neutropenic patients. J. Antimicrob. Chemother. **31**:161–169.
8. **De Wit, S., C. Rossi, J. Duchateau, A. Braitman, R. Gupta, and N. Clumeck.** 1991. Safety, tolerance and immunomodulatory effect of amphotericin B lipid complex in HIV infected subjects. Program Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 288. American Society for Microbiology, Washington, D.C.
9. **Diamond, R. D.** 1991. The growing problem of mycoses in patients infected with the human immunodeficiency virus. Rev. Infect. Dis. **13**:480–486.
10. **Fisher, M. A., G. H. Talbot, G. Maislin, B. McKeon, K. P. Tynan, and B. L. Strom.** 1989. Risk factors for amphotericin B-associated nephrotoxicity. Am. J. Med. **87**:547–552.
11. **Gondal, J. A., R. P. Swartz, and A. Rahman.** 1989. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob. Agents Chemother. **33**:1544–1548.
12. **Gouge, T. H., and V. T. Andriole.** 1971. An experimental model of amphotericin B nephrotoxicity with renal tubular acidosis. J. Lab. Clin. Med. **78**:713–724.
13. **Holleran, W. M., J. R. Wilbur, and M. W. De Gregorio.** 1985. Empiric amphotericin B therapy in patients with acute leukemia. Rev. Infect. Dis. **7**:619–624.
14. **Holmberg, K., and R. D. Meyer.** 1986. Fungal infections in patients with AIDS and AIDS-related complex. Scand. J. Infect. Dis. **18**:179–192.
15. **Janoff, A. S., L. T. Boni, M. C. Popescu, S. R. Minchey, P. R. Cullis, T. D. Madden, T. Taraschi, S. M. Gruner, E. Shyamsunder, M. W. Tate, R. Mendelsohn, and D. Bonner.** 1988. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc. Natl. Acad. Sci. USA **85**:6122–6126.
16. **Joly, V., J. Bolard, L. Saint-Julien, C. Carbon, and P. Yeni.** 1992. Influence of the phospholipid/amphotericin B ratio and the phospholipid type on the in vitro renal cell toxicity and fungicidal activity of lipid-associated amphotericin B formulations. Antimicrob. Agents Chemother. **36**:262–266.
17. **Joly, V., C. Geoffray, J. Reynes, C. Goujard, D. Méchali, and P. Yeni.** 1993. A pilot study of deoxycholate amphotericin B associated with Intralipid in the treatment of cryptococcal meningitis in patients with AIDS. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother. abstr. 814. American Society for Microbiology, Washington, D.C.
18. **Joly, V., L. Saint-Julien, C. Carbon, and P. Yeni.** 1990. Interactions of free and liposomal amphotericin B with renal proximal tubular cells in primary culture. J. Pharmacol. Exp. Ther. **255**:17–22.

18a. **Joly, V., L. Saint-Julien, C. Carbon, and P. Yeni.** 1991. In vitro renal toxicity and in vivo therapeutic efficacy in experimental *C. neoformans* meningoencephalitis in Fungizone associated to Intralipid. Program Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 578. American Society for Microbiology, Washington, D.C.

19. **Jullien, S., J. Brajtburg, and J. Bolard.** 1990. Affinity of amphotericin B for phosphotidylcholine vesicles as determinant of the in vitro cellular toxicity of liposomal preparations. Biochim. Biophys. Acta **1021**:39–45.
20. **Jullien, S., A. Vertut-Croquin, J. Brajtburg, and J. Bolard.** 1988. Circular dichroism for the determination of amphotericin B binding to liposomes. Anal. Biochem. **172**:197–202.
21. **Kan, V. L., J. E. Bennett, M. A. Amantea, M. C. Smolskis, E. McManus, D. M. Grasela, and J. W. Sherman.** 1991. Comparative safety, tolerance, and pharmacokinetics of amphotericin B lipid complex and amphotericin B deoxycholate in healthy male volunteers. J. Infect. Dis. **164**:418–421.
22. **Kirsh, R., R. Goldstein, J. Tarloff, D. Parris, J. Hook, N. Hanna, P. Bugelski, and G. Poste.** 1988. An emulsion formulation of amphotericin B improves the therapeutic index when treating systemic murine candidiasis. J. Infect. Dis. **158**:1065–1070.
23. **Larsen, R. A., M. A. E. Leal, and L. S. Chan.** 1990. Fluconazole compared with amphotericin B plus flucytosine for cryptococcal meningitis in AIDS. Ann. Intern. Med. **113**:183–187.
24. **Le Conte, P., V. Joly, L. Saint-Julien, J. M. Gillardin, C. Carbon, and P. Yeni.** 1992. Tissue distribution and antifungal effect of liposomal itraconazole in experimental cryptococcosis and pulmonary aspergillosis. Am. Rev. Respir. Dis. **145**:424–429.
25. **Legrand, P., E. Romero, B. Cohen, and J. Bolard.** 1992. Effects of aggregation and solvent on the activity of amphotericin B on human erythrocytes. Antimicrob. Agents Chemother. **36**:2518–2522.
26. **Longuet, P., V. Joly, P. Amirault, N. Seta, C. Carbon, and P. Yeni.** 1991. Limited protection by small unilamellar liposomes against the renal tubular toxicity induced by repeated amphotericin B infusions in rats. Antimicrob. Agents Chemother. **35**:1303–1308.
27. **Lopez-Berestein, G., G. P. Bodey, L. S. Frankel, and K. Mehta.** 1987. Treatment of hepatosplenic candidiasis with liposomal am-

photericin B. J. Clin. Oncol. **5**:310–317.

28. **Lopez-Berestein, G., V. Fainstein, R. M. Hopfer, K. Mehta, M. P. Sullivan, M. Keating, M. G. Rosenblum, R. Mehta, M. Luna, E. M. Hersh, J. Reuben, R. L. Juliano, and G. P. Bodey.** 1985. Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: a preliminary study. J. Infect. Dis. **151**:704–710.
29. **Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, V. Fainstein, M. Luna, E. M. Hersh, and R. Juliano.** 1983. Treatment and prophylaxis of disseminated infection due to *Candida albicans* in mice with liposome-associated amphotericin B. J. Infect. Dis. **147**:939–945.
30. **Medoff, G., J. Brajtburg, G. S. Kobayashi, and J. Bolard.** 1983. Antifungal agents useful in the therapy of systemic fungal infections. Annu. Rev. Pharmacol. Toxicol. **23**:303–330.
31. **Moreau, P., N. Milpied, N. Fayette, J. F. Ramée, and J. L. Harousseau.** 1993. Reduced renal toxicity and improved clinical tolerance of amphotericin B mixed with Intralipid compared with conventional amphotericin B in neutropenic patients. J. Antimicrob. Chemother. **31**:535–541.
32. **New, R. R. C., M. L. Chance, and S. Heath.** 1981. Antileishmanial activity of amphotericin B and other antifungal agents entrapped in liposomes. J. Antimicrob. Chemother. **8**:371–381.
33. **Proffitt, R. T., A. Satorius, S. M. Chiang, L. Sullivan, and J. P. Adler-Moore.** 1991. Pharmacology and toxicology of a liposomal formulation of amphotericin B (AmBisome) in rodents. J. Antimicrob. Chemother. **28**(Suppl. B):49–61.
34. **Sawaya, B. P., H. Weihprecht, W. R. Campbell, J. N. Lorenz, R. C. Webb, J. P. Briggs, and J. Schnermann.** 1991. Direct vasoconstriction as a possible cause for amphotericin B-induced nephrotoxicity in rats. J. Clin. Invest. **87**:2097–2107.
35. **Sculier, J. P., A. Coune, F. Meunier, C. Brassine, C. Laduron, C. Hollaert, N. Collette, C. Heymans, and J. Klastersky.** 1988. Pilot study of amphotericin B entrapped in sonicated liposomes in cancer patients with fungal infections. Eur. J. Clin. Oncol. **24**:527–538.
36. **Stamm, A. M., R. B. Diaso, W. E. Dismukes, S. Shadomy, G. A. Cloud, C. A. Bowles, G. H. Karam, and A. Espinel-Ingroff.** 1987. Toxicity of amphotericin B plus flucytosine in 194 patients with cryptococcal meningitis. Am. J. Med. **83**:236–242.
37. **Tremblay, C., M. Barza, C. Flore, and F. Szoka.** 1984. Efficacy of liposome-intercalated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob. Agents Chemother. **26**:170–173.
38. **Wasan, K. M., G. A. Brazeau, A. Keyhani, A. C. Hayman, and G. Lopez-Berestein.** 1993. Roles of liposome composition and temperature in distribution of amphotericin B in serum lipoproteins. Antimicrob. Agents Chemother. **37**:246–250.
39. **Wasan, K. M., K. Vadiei, G. Lopez-Berestein, and D. R. Luke.** 1990. Pharmacokinetics, tissue distribution and toxicity of free and liposomal amphotericin B in diabetic rats. J. Infect. Dis. **161**:562–566.