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Amphotericin B Liposomes with Prolonged Circulation in Blood: In Vitro Antifungal Activity, Toxicity, and Efficacy in Systemic Candidiasis in Leukopenic Mice

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Pegylated amphotericin B (AmB) liposomes (PEG-AmB-LIP) were compared with laboratory-prepared nonpegylated AmB liposomes (AmB-LIP), a formulation with a lipid composition the same as that in AmBisome, as well as with industrially prepared AmBisome regarding their in vitro antifungal activities, toxicities, blood residence times, and therapeutic efficacies. Killing of *Candida albicans* (>99.9%) during short-term (6-h) incubation was observed at 0.2 mg of AmB per liter for AmB desoxycholate, 0.4 mg of AmB per liter for PEG-AmB-LIP, 0.8 mg of AmB per liter for AmB-LIP, and 12.8 mg of AmB per liter for AmBisome. The maximum tolerated doses of PEG-AmB-LIP, AmB-LIP, and AmBisome were 15, 19, and >31 mg of AmB per kg of body weight, respectively. In contrast to AmB-LIP, the blood residence time of PEG-AmB-LIP was prolonged and dose independent. In a model of systemic candidiasis in leukopenic mice at a dose of 5 mg of AmB per kg, PEG-AmB-LIP was completely effective and AmB-LIP was partially effective, whereas AmBisome was not effective. AmB-LIP at 11 mg of AmB per kg was partially effective. AmBisome at 29 mg of AmB per kg was completely effective. In conclusion, the therapeutic efficacies of AmB liposomes can be improved by preparing AmB liposomes in which a substantial reduction in toxicity is achieved while antifungal activity is retained. In addition, therapeutic efficacy is favored by a prolonged residence time of AmB liposomes in blood.

There has been a steady increase in the incidence of invasive fungal infections in immunocompromised patients. These infections are among the most important causes of morbidity and mortality among patients with cancer and among other severely immunocompromised hosts. Because the overall prognosis for patients with invasive fungal infections remains poor, there is a critical need to improve the methods for treating these infections.

Parenteral administration of amphotericin B (AmB) remains the therapy of choice for most invasive fungal infections. AmB is administered as AmB desoxycholate (AmB-DOC; Fungizone), but its clinical use is limited by toxic side effects (15, 23). A promising approach to the treatment of deep systemic fungal infections is the use of AmB incorporated into liposomes or other lipid carriers (12, 16, 27). With respect to the industrially produced preparations AmBisome, AmB lipid complex, and Amphocil, it is evident that these have quite different structural and pharmacokinetic characteristics (20, 27). The relatively large structures of the AmB lipid complex as well as the small discoidal particles of Amphocil are rapidly taken up by the mononuclear phagocyte system, whereas small liposomes such as those used in the AmBisome formulation remain in the circulation for relatively prolonged periods. Until now it has not been known whether a long blood residence time is of importance for the improved efficacies of AmB liposomes.

The ability to achieve a significantly longer blood residence time of liposomes opens new ways to achieving improved delivery of antimicrobial agents to infected tissues including infections in non-mononuclear phagocyte system tissues (8). Recently, many reports have shown that hydrophilic phosphatidylethanolamine derivatives of monomethoxypolyethyleneglycols (PEG-PE) attached to the liposomes can effectively prolong their blood circulation times without the limitations of lipid dose, small particle size, or rigid lipid composition (2–5, 8, 9, 21, 22, 25, 32, 33). Such liposomes have been named sterically stabilized liposomes. The distinctive properties of sterically stabilized liposomes make them excellent candidates for many therapeutic applications.

In our laboratory it was recently shown (30) that long-circulating AmB-containing liposomes can be prepared by incorporation of polyethylene glycol 1900 (PEG)-derivatized distearoylphosphatidylethanolamine (DSPE), which is referred to as PEG-DSPE. In the present study the effects of incorporation of PEG-DSPE in AmB liposomes on in vitro antifungal activity, toxicity, and efficacy in leukopenic mice with systemic candidiasis are reported. The pegylated AmB liposome (PEG-AmB-LIP) formulation was compared with laboratory-prepared nonpegylated AmB liposomes (AmB-LIP), a formulation with a lipid composition the same as that in AmBisome, as well as with industrially prepared AmBisome.

MATERIALS AND METHODS

Materials. Antibiotic medium 3 was from Difco Laboratories (Detroit, Mich.). Sabouraud dextrose agar was from Oxoid (Basingstoke, England). AmB and AmB-DOC (Fungizone for intravenous infusion) were kindly provided by Bristol Myers-Squibb, Woerden, The Netherlands. AmB-DOC was reconstituted with distilled water to give a standard solution of 5 g of AmB per liter AmBisome, consisting of hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol), distearoylphosphatidylglycerol (DSPG), and AmB in a molar ratio of 2:1:0.8:0.4, and lipid powder, consisting of HSPC, Chol, DSPG, and AmB in a molar ratio of 2:1:0.8:0.4 in which AmB is complexed to DSPG, were both kindly provided by Vestar, Inc. (San Dimas, Calif.). HSPC, Chol, monomethoxypolyethyleneglycol 1900 succinimidyl succinate (activated PEG), and DSPE were all kindly provided by Vestar Inc. Dimethyl sulfoxide (DMSO) was from Janssen

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Chimica (Tilburg, The Netherlands). Deferoxamine mesylate (DF) was from Ciba-Geigy (Basel, Switzerland). ^{67}Ga-citrate was from Nordian (Montreal, Canada), and ^{111}In-chloride was from Frosst (Quebec, Canada). Hydroxyquinoline sulfate (oxine) and cyclophosphamide were from Sigma (St. Louis, Mo.). PEG-DSPE was synthesized as described by Blume and Cevc (9).

***Candida* strain.** *Candida albicans* ATCC 44858 was used in all of the experiments and was stored at −80°C in Todd-Hewitt broth (Difco) containing 10% (vol/vol) glycerol.

Animals. Specific-pathogen-free female BALB/c mice (ages, 12 to 20 weeks) were obtained from Iffa Credo (L'Arbresle, France).

Liposome preparation. Liposome preparations consisted of PEG-DSPE–HSPC–Chol–AmB (PEG-AmB-LIP) in a molar ratio of 0.21:1.79:1:0.32, PEG-DSPE–HSPC–Chol (PEG-PLACEBO-LIP) in a molar ratio of 0.21:1.79:1, HSPC-Chol-DSPG-AmB (AmB-LIP) in a molar ratio of 2:1:0.8:0.4, and HSPC-Chol-DSPG (PLACEBO-LIP) in a molar ratio of 2:1:0.8. AmB is very poorly soluble in chloroform-methanol (1:1; vol/vol), and therefore, as a first step in the preparation of AmB-containing liposomes, AmB was complexed to either PEG-DSPE (PEG-AmB-LIP) or DSPG (AmB-LIP). For PEG-AmB-LIP, AmB was complexed to PEG-DSPE by adding small volumes (20 to 50 μl) of 1 N HCl to a suspension of AmB and PEG-DSPE in 2 ml of chloroform-methanol (1:1; vol/vol); this was followed by heating at 65°C and vortex mixing until the solution cleared. Small volumes (10 to 15 μl) of 1 N NaOH were added; this was followed by the addition of HSPC and Chol. When precipitation of AmB was observed, small volumes of 1 N HCl were again added until the solution cleared. This lipid mixture was evaporated to dryness in a round-bottom flask at 65°C. For PEG-PLACEBO-LIP, AmB-LIP, and PLACEBO-LIP, a chloroform-methanol (1:1; vol/vol) solution of lipid mixture or lipid powder was evaporated to dryness in a round-bottom flask at 65°C. The lipid film was hydrated by vortex mixing in a buffer solution containing 10 mM sodium-succinate and 10% (wt/vol) sucrose (pH 5.5) at 65°C. For biodistribution experiments, 5 mM DF was added to this buffer. Liposomes were sonicated, resulting in an average particle size of 100 nm (range, 95 to 105 nm), as measured by dynamic light scattering (4700 system; Malvern, Malvern, United Kingdom). Biodistribution of intact liposomes was performed with liposomes radiolabeled with ^{67}Ga-DF as described by Woodle (31). The labeling resulted in the formation of a ^{67}Ga-DF complex in the aqueous interiors of the liposomes. As shown by Gabizon et al. (14), this complex is appropriate for in vivo tracing of intact liposomes because of the advantages of minimal translocation of radioactive label to plasma proteins and the rapid renal clearance rate when the label is released from the liposomes. Liposomes were separated from nonentrapped AmB, DF, or radiolabel by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) and were concentrated by using 300-kDa Microsep filters (Filtron, Breda, The Netherlands). Phospholipid concentration was determined by a phosphate assay (6). The AmB concentration was determined spectrophotometrically at 405 nm after destruction of the liposomes in DMSO-methanol (1:1; vol/vol).

AmBisome, consisting of HSPC, Chol, DSPG, and AmB in a molar ratio of 2:1:0.8:0.4, was provided as a lyophilized preparation. The powder was reconstituted according to the manufacturer's instructions with distilled water at 65°C to give a liposomal suspension containing 4 g of AmB per liter and 35 g of lipid per liter, with an average liposome particle size of 90 nm (range, 88 to 92 nm).

In vitro antifungal activities of AmB-DOC, PEG-AmB-LIP, AmB-LIP, and AmBisome. The in vitro activities of AmB-DOC, PEG-AmB-LIP, AmB-LIP, and AmBisome in terms of effective killing (>99.9%) of *C. albicans* at an inoculum of 1.3×10^7 CFU/liter during 6 h of incubation were determined as described previously (29). Briefly, a logarithmic-growth-phase culture of *C. albicans* was prepared. *C. albicans* was exposed during 6 h of incubation to twofold increasing concentrations of each agent in antibiotic medium 3, ranging from 0.05 to 25.6 mg of AmB per liter. During incubation the numbers of viable *C. albicans* were determined at 2-h intervals by making plate counts of 10-fold serial dilutions of the washed specimen on Sabouraud dextrose agar.

Determination of MTD. The toxicities of PEG-AmB-LIP, AmB-LIP, and AmBisome were measured in uninfected mice. Mice (six per group) were treated intravenously with a single dose. AmB dosages ranged from 1 to 31 mg/kg of body weight in steps of 2 mg/kg. Acute mortality was assessed directly following injection of the preparation. Blood urea nitrogen and serum creatinine levels as parameters for renal toxicity and aspartate aminotransferase and alanine aminotransferase levels as parameters for liver toxicity were determined by established methods in serum samples from mice sacrificed at 48 h after the termination of treatment. The maximum tolerated dose (MTD) was defined as the maximum dose that did not result in death or more than a threefold increase in the indices for renal and liver function compared with those for untreated mice.

Blood residence time of PEG-AmB-LIP versus that of AmB-LIP at various dosages in uninfected mice. ^{67}Ga-DF-labeled liposomes were administered intravenously at 0.5 or 5 mg of AmB per kg (equivalent to 4.7 or 47 μmol of liposomal lipid per kg, respectively) as a single dose in uninfected mice. In two separate experiments, the levels of ^{67}Ga-DF as well as those of AmB in blood were determined at various time points during 24 h after administration; three mice were used for each time point in each experiment. Blood was collected in heparinized tubes. The ^{67}Ga-DF levels in blood samples (200 μl) as well as those in the doses of the injected liposomes were counted in a gamma counter (Minaxy 5530; Packard Instruments, Downers Grove, Ill.). Additionally, at 5 mg/kg, AmB concentrations were determined in these blood samples. For determination of AmB concentrations in blood, samples from three mice were pooled and stored at −80°C until just before high-performance liquid chromatography (HPLC) analysis, which was performed as described before (29). In a separate experiment with ^{111}In-labeled syngeneic erythrocytes (18), the total blood volume in the mice was determined.

TABLE 1. MTDs of PEG-AmB-LIP, AmB-LIP, and AmBisome in uninfected mice[a]

Parameter of toxicity	MTD (mg of AmB/kg)[b]		
	PEG-AmB-LIP	AmB-LIP	AmBisome
Death after treatment	15	19	>31
Impaired renal function	>15	>19	>31
Impaired liver function	>15	>19	>31

[a] Mice were treated intravenously with a single dose. AmB dosages ranged from 1 to 31 mg/kg in steps of 2 mg/kg.

[b] Toxicity was determined in terms of death after treatment or more than threefold increase in the indices for renal function (blood urea nitrogen and serum creatinine) and liver function (aspartate aminotransferase and alanine aminotransferase) compared with those for placebo-treated mice determined 48 h after the termination of treatment.

Efficacies of PEG-AmB-LIP, AmB-LIP, and AmBisome in leukopenic mice infected with *C. albicans*. Leukopenia was induced by intraperitoneal administration of cyclophosphamide at 100 mg/kg 4 days before *C. albicans* inoculation. This was followed by the administration of an additional dose of 75 mg/kg on the day of inoculation and the same dose at 3-day intervals thereafter. This treatment resulted in persistent granulocytopenia ($<0.1 \times 10^9$/liter) from the time of *C. albicans* inoculation to the termination of the study. Leukopenic mice were infected by inoculation of 3×10^4 CFU *C. albicans* into the tail vein. PEG-AmB-LIP, AmB-LIP, AmBisome, and placebo were each administered intravenously as a single dose at 20 h after *C. albicans* inoculation at doses corresponding to their MTDs. AmB-LIP and AmBisome were also administered at a dose which was equivalent to the MTD of PEG-AmB-LIP. Just before treatment and at 48 h as well as 6 days after treatment, the surviving mice were sacrificed. The kidneys, liver, spleen, and lungs were removed and processed for the determination of viable counts as described previously (29). The following criteria were used to assess the efficacy of treatment: survival of mice up to 6 days after treatment, a statistically significant reduction in the numbers of CFU of *C. albicans* in the kidneys at 48 h after treatment compared with the numbers at the time of treatment, and prevention of a significant increase in the numbers of CFU of *C. albicans* in the kidneys at 6 days after treatment compared with the numbers at 48 h after treatment (equating to a relapse of infection).

Statistical analysis. Results were expressed as the geometric means ± standard deviations. Differences in the numbers of CFU of *C. albicans* between the various treatment groups were analyzed by the Mann-Whitney test.

RESULTS

In vitro antifungal activities of AmB-DOC, PEG-AmB-LIP, AmB-LIP, and AmBisome. For AmB-DOC, PEG-AmB-LIP, AmB-LIP, and AmBisome the minimal AmB concentrations required to kill >99.9% of the initial *C. albicans* inoculum within 6 h of incubation were determined. With AmB-DOC, PEG-AmB-LIP, and AmB-LIP, effective killing was obtained with 0.2, 0.4, and 0.8 mg of AmB per liter, respectively. With AmBisome, on the other hand, a concentration of 12.8 mg of AmB per liter was required to produce the same fungicidal activity.

MTDs of PEG-AmB-LIP, AmB-LIP, and AmBisome in uninfected mice. The MTDs of PEG-AmB-LIP, AmB-LIP, and AmBisome are presented in Table 1. With PEG-AmB-LIP the MTD with no death after treatment was 15 mg/kg. At this dose renal or liver toxicity was not yet observed. For AmB-LIP (a formulation with a lipid composition the same as that in AmBisome), death during treatment was shown at doses greater than 19 mg/kg. At this dose no renal or liver toxicity was seen. With AmBisome no toxicity was observed in terms of death or impairments in renal or liver function up to a dose of 31 mg/kg.

Blood residence time of PEG-AmB-LIP versus that of AmB-LIP at various doses in uninfected mice. The levels of ^{67}Ga-DF in blood following administration of radiolabeled PEG-AmB-

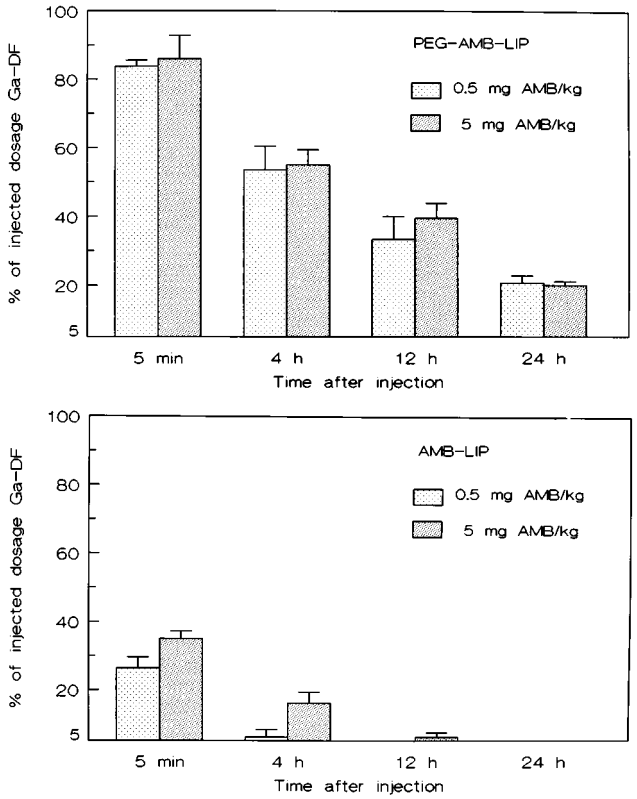

FIG. 1. Levels of ^{67}Ga-DF in blood after administration of radiolabeled PEG-AmB-LIP and AmB-LIP to uninfected mice at various doses. A single dose of 0.5 mg of AmB per kg (4.7 μmol of liposomal lipid per kg) or 5 mg of AmB per kg (47 μmol of liposomal lipid per kg) was administered intravenously. Data are expressed as the mean ± standard deviation percentage of the injected dose of ^{67}Ga-DF in two separate experiments, with three mice in each experiment (n = 6). The lack of error bars indicates that the range is smaller than the datum points.

LIP and AmB-LIP at doses of 0.5 mg of AmB per kg (4.7 μmol of lipid per kg) and 5 mg of AmB per kg (47 μmol of lipid per kg) are given in Fig. 1. ^{67}Ga-DF was used as a marker for intact liposomes. With PEG-AmB-LIP independent of dose a prolonged blood residence time of intact liposomes was observed, with 20% of the injected dose of liposomes still circulating at 24 h after administration. For AmB-LIP it was shown that within 5 min after administration the levels of intact liposomes dropped to 26 and 35% of the injected dose of liposomes at doses of 0.5 and 5 mg of AmB per kg, respectively. After this initial drop only at the highest dose of 5 mg of AmB per kg a prolonged blood residence time was observed, with 6% of the injected dose circulating 12 h after administration. At the lower dose of 0.5 mg of AmB per kg, the levels of liposomes in blood declined to 6% of injected dose within 4 h after administration.

The levels of both ^{67}Ga-DF and AmB in blood following administration of radiolabeled PEG-AmB-LIP and AmB-LIP at 5 mg of AmB per kg were determined. For both types of liposomes, the levels of AmB declined more rapidly than the levels of intact liposomes during circulation (data not shown).

Efficacies of PEG-AmB-LIP, AmB-LIP, and AmBisome in leukopenic mice infected with *C. albicans*. In leukopenic mice infected with *C. albicans* the infection is disseminated to the kidney, liver, spleen, and lung, and untreated mice die between 24 h and 8 days after *C. albicans* inoculation. In these infected mice, the MTDs of PEG-AmB-LIP, AmB-LIP, and AmBisome in terms of acute death were 5, 11, and 29 mg of AmB per kg, respectively. The effect of treatment on the survival of the animals and the growth of *C. albicans* in the kidney is presented in Table 2. An increase in the numbers of CFU of *C. albicans* was observed after placebo treatment, resulting in the deaths of the animals. Because the placebo-treated animals died, the numbers of *C. albicans* organisms in the kidneys of these mice could not be compared with those in the kidneys of AmB-treated mice. Treatment with PEG-AmB-LIP at the MTD (5 mg of AmB per kg) was completely effective: 100% survival of mice was observed, as was a significant reduction in the numbers of *C. albicans* organisms in the liver ($P \leq 0.01$), spleen ($P \leq 0.001$), and lung ($P \leq 0.025$) (data not shown). The numbers of *C. albicans* organisms in the kidney were significantly reduced at 48 h after treatment ($P \leq 0.001$), and relapse of infection was prevented; the numbers of *C. albicans* organisms were significantly reduced even further at 6 days after treatment ($P \leq 0.01$). Treatment with AmB-LIP at an equivalent dose (5 mg of AmB per kg) was partially effective: survival of all animals was observed, and there was also a signif-

TABLE 2. Effect of treatment on survival of mice and growth of *C. albicans* in the kidneys of leukopenic mice[a]

Treatment[b]	Dose (mg/kg)	Log_{10} CFU/kidney at time of treatment[b,c]	48 h after treatment[d]		6 days after treatment[d]		
			% Survival	Log_{10} CFU/kidney in surviving mice[c]	% Survival	Log_{10} CFU/kidney in surviving mice[c]	No. of mice with sterile kidney/no. of surviving mice
None		4.16 ± 0.23					
PEG-AmB-LIP	5		100	3.05 ± 0.76[e]	100	1.99 ± 0.42[f]	0/10
AmB-LIP	5		100	4.48 ± 0.83	100	4.04 ± 0.91	0/10
AmB-LIP	11		100	4.27 ± 0.20	100	2.54 ± 0.50[f]	0/10
AmBisome	5		70	5.26 ± 0.57	40	3.92 ± 0.51	0/4
AmBisome	29		100	2.60 ± 0.32[e]	100	1.01 ± 1.72[f]	2/10
PEG-PLACEBO-LIP			40	6.38 ± 0.22	30	6.12 ± 0.39	0/3
PLACEBO-LIP			50	6.56 ± 0.21	10	7.39	0/1

[a] Leukopenic mice were inoculated intravenously at zero time with 3×10^4 CFU of *C. albicans*.
[b] PEG-AmB-LIP, AmB-LIP, AmBisome, PEG-PLACEBO-LIP, and PLACEBO-LIP were administered intravenously 20 h after inoculation.
[c] Each value represents the geometric mean ± standard deviation.
[d] Effect of treatment was determined at 48 h (n = 10) as well as 6 days (n = 10) after treatment.
[e] $P \leq 0.01$ compared with the number of CFU at 20 h after inoculation (time of treatment).
[f] $P \leq 0.01$ compared with the number of CFU at 48 h after treatment.

icant reduction in the numbers of *C. albicans* organisms in the liver ($P \leq 0.025$), spleen ($P \leq 0.001$), and lung ($P \leq 0.001$) (data not shown); however, in the kidney the growth of *C. albicans* was only inhibited, and the numbers of *C. albicans* organisms were not significantly reduced. Although an increase in the dose of AmB-LIP up to the MTD (11 mg of AmB per kg) resulted in a slight improvement in efficacy compared with a dose of 5 mg of AmB per kg, it did not result in complete efficacy of treatment, as seen with PEG-AmB-LIP at 5 mg of AmB per kg; with 11 mg of AmB per kg the numbers of *C. albicans* organisms in the kidney were significantly reduced compared with the numbers at the time of treatment only at 6 days after treatment. AmBisome at a dose of 5 mg of AmB per kg was not effective, because the animals died after treatment. AmBisome at the MTD (29 mg of AmB per kg) was as effective as PEG-AmB-LIP at 5 mg of AmB per kg.

DISCUSSION

With respect to the in vitro antifungal activity of the AmB liposome formulations against *C. albicans*, it is derived from the results of the present study that after encapsulation of AmB in PEG-AmB-LIP or AmB-LIP the high degree of antifungal activity seen with AmB-DOC is almost fully retained, whereas entrapment of AmB in AmBisome is accompanied by a substantial loss of antifungal activity. Although it was previously shown that AmB-DOC and AmBisome were equally active after long-term exposure (MIC, minimum fungicidal concentration) (7, 29), the activity of AmBisome during short-term exposure was significantly less than that of AmB-DOC (29). Recently, it was reported (24) that even after long-term exposure (24 h) AmBisome was four to eight times less active than AmB-DOC against *C. albicans*.

Comparison of the MTD of the industrially prepared AmBisome in healthy mice (>31 mg of AmB per kg) and the MTD of a laboratory-prepared AmB liposome formulation (AmB-LIP) with the same lipid composition (19 mg of AmB per kg) revealed that the method of preparation greatly influences the toxicity of the AmB liposome formulation. Because the MTD of AmB-DOC in terms of the number of acute deaths after a single-dose treatment is 0.8 mg of AmB per kg (unpublished data), encapsulation of AmB in AmB-LIP still substantially reduced the toxicity of AmB. To the same extent, this also applied to PEG-AmB-LIP (MTD, 15 mg of AmB/kg).

The prolonged residence time of PEG-AmB-LIP compared with that of AmB-LIP was previously demonstrated at a dose of 9 mg of AmB per kg (corresponding to 85 μmol of lipid per kg) (30). A valuable asset of the PEG-PE-containing liposomes is that they show dose-independent pharmacokinetics (4, 5, 25, 33). Taking together the data from our present study and our previous study (30), we conclude that for PEG-AmB-LIP a prolonged blood residence time does not depend on the lipid dose for a dose range of 4.7 to 85 μmol of lipid per kg. These results are in good agreement with those reported elsewhere for PEG-PE liposomes with similar particle sizes (4, 5, 25, 33), demonstrating dose-independent blood circulation times for dose ranges of 0.5 to 500 μmol/kg in mice (4, 25, 33) and 3 to 100 μmol/kg (5, 33) in rats. On the contrary, as reported previously by Allen and Hansen (4), the blood residence times of small liposomes of approximately 100 nm in diameter with a rigid liposomal bilayer and without surface modifications are dependent on the lipid dose given. In the present study it was shown that the blood residence time of AmB-LIP depends on the lipid dose, which is in accordance with the previous observations (4). For AmBisome, the dose-dependent blood circulation of AmB was already reported previously (13, 17, 26, 28).

The extent to which a prolonged residence time of liposomal AmB is of importance for improved therapeutic efficacy was studied in a model of systemic candidiasis in leukopenic mice after a single-dose treatment. The efficacies of PEG-AmB-LIP, AmB-LIP, and AmBisome were compared by using equivalent doses of 5 mg of AmB per kg (which is the MTD of PEG-AmB-LIP in leukopenic infected mice). At this dose treatment with PEG-AmB-LIP was completely effective, but treatment with AmB-LIP was only partially effective. We conclude that therapeutic efficacy in this model of deep systemic candidiasis is favored by the prolonged residence time of AmB liposomes in the blood compartment. Whether this is due to the sustained release of AmB during circulation in the blood or increased localization of AmB liposomes at the sites of infection, as was described by others for a localized site of infection (8) or a solid tumor (19, 25), is not yet clear. AmBisome at 5 mg of AmB per kg was not effective at all. Additionally, AmB-LIP and AmBisome were both studied at their own MTDs. Complete efficacy of treatment could not be achieved with AmB-LIP, not even after an increase in the dose to 11 mg of AmB per kg (the MTD). AmBisome at 29 mg of AmB per kg (the MTD) was as effective as treatment with PEG-AmB-LIP at the almost sixfold lower dose of 5 mg of AmB per kg. Until now, the efficacy of AmBisome has been compared with that of AmB-DOC in several models of fungal infections (1, 10, 11, 13, 17, 29). From those studies it can be concluded that, depending on the model of fungal infection, the immune status of the host, and the parameter of efficacy used, the antifungal activity of AmBisome is either somewhat less than or equal to that of AmB-DOC with equivalent doses of AmB. However, treatment with AmB-DOC is restricted by acute toxicity. By using AmBisome, much higher doses are tolerated, resulting in improved antifungal efficacy, even against severe infections in immunocompromised animals. Only recently (24), the interpretation of the data from these experimental studies on AmBisome has been critically discussed. The investigators rightly note that a difference in toxicity, and thereby in the therapeutic index, between AmBisome and AmB-DOC has always been claimed on the basis of the acute toxicity of rapidly injected AmB-DOC in small laboratory animals. As in patients, AmB-DOC is not administered as an intravenous bolus but is administered by slow infusion; the investigators studied the therapeutic efficacies of both AmB-DOC and AmBisome in localized and systemic murine candidiases, with AmB-DOC administered at high doses in multiple fractions. The observation that AmBisome was less active than high doses of AmB-DOC indicates that by entrapment of AmB in liposomes, therapeutic efficacy might be reduced. From the present study it is also clear that with AmBisome a reduction in toxicity is concomitant with a reduction in antifungal activity, and thereby in antifungal efficacy in vivo. At an equivalent dose, AmB-LIP, a formulation with which somewhat less reduction in toxicity occurs together with retained antifungal activity, shows better antifungal efficacy in vivo.

By combining the data on toxicity, antifungal activity, blood residence time, and efficacy of treatment, two important conclusions can be drawn. First, from a comparison between laboratory-prepared AmB-LIP with industrially prepared AmBisome, it is clear that the method of preparation of an AmB liposome formulation greatly influences the toxicity and antifungal activity, and thereby the antifungal efficacy, in vivo. It should be stressed that a maximal reduction of AmB toxicity results in a concomitant loss of antifungal activity and efficacy. Therefore, for optimization of the preparation of AmB liposomes it is important to focus on both a reduction in AmB toxicity and retained antifungal activity. This is achieved in

both AmB-LIP and PEG-AmB-LIP. The second conclusion derived by comparing PEG-AmB-LIP and AmB-LIP is that therapeutic efficacy is favored by a prolonged residence time of AmB liposomes in the blood compartment.

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