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Superior Efficacy of Liposomal Amphotericin B with Prolonged Circulation in Blood in the Treatment of Severe Candidiasis in Leukopenic Mice

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In leukopenic mice with severe systemic candidiasis, single-dose treatment (5 mg of amphotericin B [AMB]/kg of body weight) with long-circulating polyethylene glycol-coated AMB liposomes (PEG-AMB-LIP) resulted in zero mortality and a significant reduction in the number of viable *Candida albicans* in the kidney, whereas 70% mortality was seen in mice treated with five daily doses of AmBisome (5 mg of AMB/kg · day). When the first of five daily doses of AmBisome was combined with a single low dose of Fungizone (0.1 mg of AMB/kg), the efficacy was equal to that of PEG-AMB-LIP.

Fungizone (amphotericin B [AMB] deoxycholate) remains the therapy of choice for most invasive fungal infections, but the use of this drug is significantly limited by its toxic side effects. Lipid formulations of AMB have been developed by the pharmaceutical industry, with the primary aim of a reduction of AMB's toxicity. Strikingly, it is not well recognized that the reduction of AMB's toxicity following lipid formulation seems to be associated with a substantial reduction of the drug's direct antifungal activity. It has been previously reported that the in vitro activity of AmBisome during short-term exposure of *Candida albicans* was significantly less than that of Fungizone (4, 5, 7). Furthermore, various in vivo studies have clearly demonstrated that high dosages of AMB-lipid formulations are often needed for a treatment to be effective (3, 5, 7). Recently, we were able to show that it is now possible, by using a lipid formulation, to substantially reduce the toxicity of AMB without reducing its direct antifungal activity (7). A new type of liposomal AMB in which AMB is complexed to a hydrophilic phospholipid derivative of polyethylene glycol (PEG-AMB-LIP) was prepared in our laboratory. The PEG-AMB-LIP formulation shows three characteristics that are expected to be important for improved antifungal efficacy (6, 7): low toxicity, high direct antifungal activity, and prolonged circulation time of intact liposomes in blood. Prolonged blood residence of intact liposomes may be important for increased accumulation of liposomal AMB at sites of fungal infection outside the mononuclear phagocyte system, such as the kidney and lung (1, 2).

In a previous study with our model of severe invasive *C. albicans* infection, it was shown that treatment with a single dose of PEG-AMB-LIP (5 mg of AMB/kg of body weight) resulted in decreasing numbers of viable *C. albicans* in the kidney within a short period of time after inoculation with this organism. This effect could not be achieved with AmBisome at the same dosage; however, a much higher dosage of AmBisome (29 mg of AMB/kg) was as effective as PEG-AMB-LIP. In the present study, the significance of the high direct antifungal activity of PEG-AMB-LIP for the treatment of acute severe systemic candidiasis is further explored. The effect of early or delayed treatment with a single dose of PEG-AMB-LIP was compared with that of single- or multidose treatment with AmBisome. Furthermore, the potential benefit of adding a single low dose of Fungizone to the first dose of AmBisome was investigated.

Sabouraud dextrose agar was from Unipath Ltd. (Basingstoke, England). AMB and Fungizone were kindly provided by Bristol Myers-Squibb (Woerden, The Netherlands). AmBisome was from NeXstar Pharmaceuticals, Inc. (San Dimas, Calif.). Hydrogenated soybean phosphatidylcholine (HSPC) and a polyethylene glycol 1900 derivative of distearoylphosphatidylethanolamine (PEG-DSPE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, Ala.). Dimethyl sulfoxide was from Janssen Chimica (Tilburg, The Netherlands). Cyclophosphamide and cholesterol (Chol) were from Sigma (St. Louis, Mo.). Chloroform and methanol were from Merck (Darmstadt, Germany). *C. albicans* ATCC 44858 was used (5). Specific-pathogen-free 12- to 20-week-old female BALB/c mice were obtained from Iffa Credo (L'Arbresle, France).

PEG-DSPE–HSPC–Chol–AMB in a molar ratio of 0.21:1.79:1:0.32 (PEG-AMB-LIP) and placebo liposomes (devoid of AMB) were prepared as described previously (6, 7). AmBisome, consisting of HSPC-Chol-distearoylphosphatidylglycerol-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.

Leukopenic mice were infected with *C. albicans* as previously described (7). The treatment regimens studied were as follows: (i) single-dose PEG-AMB-LIP, (ii) single- or multidose AmBisome, (iii) multidose AmBisome combined with a single dose of Fungizone, and (iv) single-dose Fungizone.

(i) Single-dose PEG-AMB-LIP. PEG-AMB-LIP was administered intravenously (i.v.) as a single dose of the maximum tolerated dosage of 5 mg of AMB/kg (7). Treatment was started at either 6, 16, or 20 h after *C. albicans* inoculation.

(ii) Single- or multidose AmBisome. AmBisome was administered i.v. either as a single dose of 5 mg of AMB/kg or daily at 5 mg of AMB/kg · day for three or five consecutive days. Treatment was started at either 6, 16, or 20 h after *C. albicans* inoculation.

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TABLE 1. Effect of early or delayed treatment with a single dose of PEG-AMB-LIP versus single- or multidose AmBisome on survival of leukopenic mice[a] and growth of *C. albicans* in the kidney

Time of start of treatment (h postinoculation)	Treatment[b]	Daily dose (mg/kg)	No. of doses	Log_{10} CFU/kidney in surviving mice on day[c]:		
				0	3	7
6	None			3.20 ± 0.16		
	Placebo				5.94 ± 0.11 (50)	6.86 ± 0.07 (30)
	PEG-AMB-LIP	5	1		1.04 ± 0.39[d] (100	1.16 ± 0.70[d] (100)
	AmBisome	5	1		3.28 ± 0.20 (100)	3.47 ± 0.41 (100)
	AmBisome	5	3		3.08 ± 0.26 (100)	3.46 ± 0.57 (100)
	AmBisome	5	5		2.90 ± 0.44 (100)	2.63 ± 0.46[d] (100)
16	None			4.01 ± 0.22		
	PEG-AMB-LIP	5	1		2.29 ± 0.42[d] (100)	1.35 ± 0.51[d,e] (100)
	AmBisome	5	5		4.79 ± 0.40 (90)	3.43 ± 0.38 (90)
20	None			4.34 ± 0.19		
	PEG-AMB-LIP	5	1		2.88 ± 0.38[d] (100)	2.31 ± 0.19[d,e] (100)
	AmBisome	5	5		5.39 ± 0.45 (60)	4.57 ± 1.11 (30)

[a] Leukopenic mice were inoculated i.v. at time zero with 3×10^4 CFU of *C. albicans*; untreated mice died between 24 h and 8 days after *C. albicans* inoculation.
[b] PEG-AMB-LIP, AmBisome, and the placebo were administered i.v.
[c] The effect of treatment was determined at day 3 ($n = 10$) as well as day 7 ($n = 10$) after inoculation. Each value represents the geometric mean ± the standard deviation. The numbers in parentheses indicate the percentages of surviving mice. Values at day 0 are log_{10} CFU/kidney at the start of treatment.
[d] $P \leq 0.002$ compared with the number of CFU at the start of treatment.
[e] $P \leq 0.002$ compared with the number of CFU at day 3 after inoculation.

(iii) Multidose AmBisome combined with a single dose of Fungizone. The first of five daily doses of AmBisome (5 mg of AMB/kg · day) was administered at 20 h after inoculation with *C. albicans* and combined with a single i.v. dose of the maximum tolerated dosage (0.1 mg of AMB/kg) of Fungizone (5). The two agents were administered separately, with the first dose of AmBisome being given directly after Fungizone.

(iv) Single-dose Fungizone. Fungizone, at 0.1 mg of AMB/kg, was administered i.v. 20 h after *C. albicans* inoculation.

The efficacy of treatment was assessed as previously described (7). In the present study, only the numbers of viable *C. albicans* in the kidneys were determined, since it was previously shown (5, 7) that in this animal model the kidney is the most severely infected organ. Differences in *C. albicans* CFU between the various treatment groups were analyzed by the Mann-Whitney test.

By increasing the delay of the start of treatment, the efficacy of treatment in relation to the severity of infection could be investigated. This was reflected in increasing numbers ($P \leq 0.01$) of viable *C. albicans* in the kidneys at the start of treatment (Table 1). The effect of early or delayed treatment with a single dose of PEG-AMB-LIP versus single- or multidose AmBisome treatment is presented in Table 1. When AmBisome treatment was started 6 h after *C. albicans* inoculation, five daily doses (5 mg/kg · day) were needed to significantly reduce the number of viable *C. albicans* at 7 days after inoculation compared to the number at the start of treatment. The rate of response to this therapy was, thus, relatively low.

In this model of acute severe systemic candidiasis, in which untreated animals start to die 24 h after *C. albicans* inoculation, the efficacy of a 5-day AmBisome treatment regimen after a delay in the start of treatment was questioned. When treatment was delayed until 16 h after inoculation, 10% of the animals died after five daily doses of AmBisome. The further delay in treatment to 20 h after inoculation resulted in an increase in mortality to 70%. In sharp contrast, a single dose of PEG-AMB-LIP administered at 20 h after inoculation still resulted in zero mortality and a significant reduction in the number of viable *C. albicans* in the kidney. The present study shows that the high efficacy observed for PEG-AMB-LIP at a single-dose administration could not be matched by a multidose regimen of AmBisome, although the latter results in prolonged blood residence. Probably the high direct antifungal activity of AMB is of major importance in the successful treatment of severely infected animals with PEG-AMB-LIP: treatment with PEG-AMB-LIP resulted in a rapid response in terms of decreasing numbers of viable *C. albicans* in the kidney, whereas AmBisome treatment did not.

Apparently, AMB in PEG-AMB-LIP is readily available for immediate antifungal activity. To gain more insight into the mechanism by which PEG-AMB-LIP exerts its rapid antifungal effect, factors important for the specific transfer of AMB from PEG-AMB-LIP to fungal cells are presently under investigation. The possibility of a slight release of AMB from PEG-AMB-LIP in the blood after i.v. administration cannot be ex-

TABLE 2. Effect of multidose AmBisome treatment with or without a single dose of Fungizone on survival of leukopenic mice[a] and growth of *C. albicans* in the kidney

Treatment[b]	Daily dose (mg/kg)	No. of doses	Log_{10} CFU/kidney in surviving mice on day[c]:	
			3	7
AmBisome	5	5	5.39 ± 0.45 (60)	4.57 ± 1.11 (30)
Fungizone + AmBisome	0.1 + 5	1 + 5	3.00 ± 0.17[d] (100)	2.06 ± 0.31[d,e] (100)
Fungizone	0.1	1	5.67 ± 0.12 (40)	6.38 ± 0.41 (40)

[a] Leukopenic mice were inoculated i.v. at time zero with 3×10^4 CFU of *C. albicans*; untreated mice died between 24 h and 8 days after *C. albicans* inoculation.
[b] AmBisome and Fungizone were administered i.v. at 20 h after *C. albicans* inoculations. In the combination treatment, both agents were administered separately, with the first dose of AmBisome being given directly after Fungizone.
[c] The effect of treatment was determined at day 3 ($n = 10$) as well as day 7 ($n = 10$) after inoculation. The log_{10} CFU/kidney at the start of treatment (day 0) was 4.34 ± 0.19. Each value represents the geometric mean ± the standard deviation. The numbers in parentheses indicate the percentages of surviving mice (see also the legend to Table 1).
[d] $P \leq 0.002$ compared with the number of CFU at the start of treatment.
[e] $P \leq 0.002$ compared with the number of CFU at day 3 after inoculation.

cluded, although it has been previously shown that during circulation AMB is tightly associated with the liposomes in PEG-AMB-LIP (6).

The second part of the study involved investigating whether a lack of direct availability of AMB in AmBisome might be responsible for the low efficacy of that drug in severely infected mice. At 20 h after *C. albicans* inoculation, the first of five daily doses of AmBisome was combined with a single low dose of Fungizone (0.1 mg of AMB/kg) (Table 2). Strikingly, the efficacy of this combined treatment was equal to that of PEG-AMB-LIP (Tables 1 and 2). After receiving a single low dose of Fungizone alone, the animals died (Table 2). The observation that in severe invasive fungal infection the efficacy of treatment with AmBisome can be greatly improved by addition of a single low dose of Fungizone in the early phase of treatment may be of great clinical significance.

REFERENCES

1. **Bakker-Woudenberg, I. A. J. M., A. F. Lokerse, M. T. ten Kate, J. W. Mouton, M. C. Woodle, and G. Storm.** 1993. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J. Infect. Dis. **168:**164–171.
2. **Bakker-Woudenberg, I. A. J. M., M. T. ten Kate, L. E. T. Stearne-Cullen, and M. C. Woodle.** 1995. Efficacy of gentamicin or ceftazidime entrapped in liposomes with prolonged blood circulation and enhanced localization in *Klebsiella pneumoniae* infected lung tissue. J. Infect. Dis. **171:**938–947.
3. **Leenders, A. C. A. P., and S. De Marie.** 1996. The use of lipid formulations of amphotericin B for systemic fungal infections. Leukemia **10:**1570–1575.
4. **Legrand, P., M. Chéron, L. Leroy, and J. Bolard.** 1997. Release of amphotericin B from delivery systems and its action against fungal and mammalian cells. J. Drug Targeting **4:**311–319.
5. **van Etten, E. W. M., C. van den Heuvel-De Groot, and I. A. J. M. Bakker-Woudenberg.** 1993. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J. Antimicrob. Chemother. **32:**723–739.
6. **van Etten, E. W. M., W. van Vianen, R. H. G. Tijhuis, G. Storm, and I. A. J. M. Bakker-Woudenberg.** 1995. Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice. J. Control. Release **37:**123–129.
7. **van Etten, E. W. M., M. T. ten Kate, L. E. T. Stearne, and I. A. J. M. Bakker-Woudenberg.** 1995. Amphotericin B liposomes with prolonged circulation in blood: in vitro antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice. Antimicrob. Agents Chemother. **39:** 1954–1958.