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# Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice

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**Abstract**

In this study it was investigated whether long-circulating amphotericin B (AMB) containing liposomes could be prepared by incorporation of polyethylene glycol (1900) derivatized distearoylphosphatidylethanolamine (PEG-DSPE), and whether the incorporation of PEG-DSPE affected toxicity and biodistribution of the preparation in mice. Toxicity of two formulations of liposomes containing both PEG-DSPE and AMB (PEG-AMB-LIP, types 1 and 2) was compared with that of AMB-liposomes without PEG-DSPE (AMB-LIP) as well as that of MB-deoxycholate (AMB-DOC). The maximum tolerated dosage (MTD) of AMB-DOC, expressed in terms of death during treatment for 5 consecutive days or significant increases in the parameters used to monitor renal and hepatic functions, was 0.8 mg/kg per day. AMB-LIP were the least toxic, the MTD being 11 mg/kg per day. The formulation with AMB complexed to DSPG (PEG-AMB-LIP type 1) was as toxic as AMB-DOC. This PEG-AMB-LIP formulation was omitted from further studies on biodistribution. With AMB complexed to PEG-DSPE (PEG-AMB-LIP type 2) toxicity was substantially reduced, resulting in a MTD of 9 mg/kg per day. Biodistribution of radiolabeled PEG-AMB-LIP type 2 was compared with that of AMB-LIP. Blood residence time of PEG-AMB-LIP type 2 was prolonged as compared to AMB-LIP; For PEG-AMB-LIP type 2 at 24 h after administration 30% of the injected dosage of AMB in intact liposomes was circulating versus 6% for AMB-LIP.

*Keywords:* Amphotericin B; Biodistribution; Toxicity; Liposome; Poly(ethylene glycol)

## 1. Introduction

The therapy of choice for most invasive fungal infections remains amphotericin B (AMB), administered as amphotericin-deoxycholate (AMB-DOC or Fungizone®). However, its clinical use is limited by toxic side effects [1]. A promising approach in the treatment of deep systemic fungal infections is AMB incorporated in liposomes or other lipid-carriers [1–3]. With respect to the industrially produced preparations AmBisome®, ABLC (AMB lipid complex) and Amphocil® it is evident that these have quite different structural and pharmacokinetic characteristics [4]. The

Abbreviations: AMB, amphotericin B; AMB-DOC, amphotericin B-deoxycholate; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BUN, blood urea nitrogen; CREAT, serum creatinine; DF, deferoxamine mesylate; Chol, cholesterol; DSPG, distearoylphosphatidylglcerol; HSPC, hydrogenated soybean phosphatidylcholine; MPS, mononuclear phagocyte system; PEG-DSPE, polyethylene glycol(1900) derivatized distearoylphosphatidylethanolamine

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relatively large structures of ABLC, as well as the small discoidal particles of Amphocil® are rapidly taken up by the mononuclear phagocyte system (MPS), whereas small liposomes as in AmBisome® remain in the circulation for relatively prolonged periods. Until now it is not known whether blood residence time is an important variable influencing the therapeutic index of AMB-liposomes.

The ability to achieve a significantly longer blood residence time of liposomes creates new opportunities for improving the delivery of antimicrobial agents to infected tissues [5,6]. Recently, many reports have shown that hydrophilic phosphatidylethanolamine derivatives of monomethoxy polyethylene glycols (PEG-PE) attached to the liposomes enhance blood circulation time without the limitations of lipid dose or rigid composition [6–19]. Such long-circulating liposomes have been named sterically stabilized liposomes.

As the effects of inclusion of PEG-DSPE on the therapeutic index of AMB-liposomes are not yet reported, we have investigated whether long-circulating AMB containing liposomes can be prepared by incorporation of PEG-DSPE, and whether the incorporation of PEG-DSPE affects the toxicity and biodistribution of the preparation.

Successful liposomal encapsulation of AMB requires that AmB is complexed, e.g. to DSPG, as in the current AmBisome® formulation. We have demonstrated that complexation of AMB to PEG-DSPE can also be achieved (manuscript in preparation). In this study the preparation of two different formulations of liposomes containing both PEG-DSPE and AMB is described. PEG-AMB-LIP type 1, in which PEG-DSPE was added to AMB-liposomes with AMB complexed to DSPG, as well as PEG-AMB-LIP type 2, in which AMB was complexed to PEG-DSPE and DSPG was omitted from the preparation, were prepared. These pegylated formulations were compared with non-pegylated AMB-liposomes (referred to as AMB-LIP) regarding toxicity and biodistribution.

## 2. Materials and methods

### 2.1. Materials

AMB and AMB-DOC (Fungizone® for intravenous infusion) were kindly provided by Bristol Myers-Squibb, The Netherlands. AMB-DOC was reconstituted according to the manufacturer's instructions with distilled water to give a standard solution of 5 g AMB/l. Lipid powder consisting of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4 in which AMB is complexed to DSPG was kindly provided by Vestar Inc. (San Dimas, CA). Hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol), monomethoxy polyethylene glycol (1900) succinimidyl succinate (activated PEG), distearoylphosphatidylethanolamine (DSPE), were all kindly provided by Vestar Inc. Dimethyl sulfoxide (DMSO) was from Janssen Chimica (Tilburg, The Netherlands). Deferoxamine mesylate (DF) was from CibaGeigy (Basel, Switzerland). $^{67}$Ga-citrate was from Nordian (Montreal, Canada), $^{111}$In-chloride from Frosst (Quebec, Canada). Hydroxyquinoline sulfate (oxine) was from Sigma (St. Louis, MO). PEG (1900) derivative of DSPE (PEG-DSPE) was synthesized as described by Blume and Cevc [11].

### 2.2. Liposome preparation

Liposome preparations consisted of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4 (AMB-LIP), HSPC:Chol:DSPG in a molar ratio of 2:1:0.8 (PLACEBO-LIP), PEG-DSPE:HSPC:Chol: DSPG: AMB in a molar ratio of 0.29:2:1:0.8:0.4 (PEG-AMB-LIP type 1), PEG-DSPE:HSPC:Chol:AMB in a molar ratio of 0.21:1.79:1:0.32 (PEG-AMB-LIP type 2), and PEG-DSPE:HSPCChol in a molar ratio of 0.21:1.79:1 (PEG-PLACEBO-LIP type 2). AMB is very poorly soluble in chloroform/methanol (1:1, v/v), and therefore as a first step in the preparation of AMB containing liposomes AMB was complexed to either DSPG (AMB-LIP and PEG-AMB-LIP type 1) or to PEG-DSPE (PEG-AMB-LIP type 2). For AMB-LIP, PLACEBO-LIP, PEG-AMB-LIP type 1, and PEG-PLACEBO-LIP type 2 a chloroform/methanol (1:1, v/v) solution of lipid powder or lipid mixture was evaporated to dryness in a round-bottom flask at 65°C. For PEG-AMB-LIP type 2, AMB was complexed to PEG-DSPE by adding small volumes (20–50 μl) of 1 N HCl to a suspension of AMB and PEG-DSPE in 2 ml chloroform/methanol (1:1, v/v), followed by heating at 65°C and vortex mixing until the solution cleared. Small volumes (10–15 μl) of 1 N NaOH were added, followed by addition of HSPC and

Chol. When precipitation of AMB was observed, again small volumes of 1 N HCl were added until the solution cleared. This lipid mixture 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% (w/v) 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 95–105 nm), as measured by dynamic light scattering (Malvern 4700 system, Malvern, UK). Biodistribution of intact liposomes was performed using liposomes radiolabeled with $^{67}$Ga-DF as described by Woodle [20]. The labeling resulted in formation of a $^{67}$Ga-DF complex in the aqueous interior of liposomes. As shown by Gabizon et al. [21], 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 extracellularly. Liposomes were separated from non-entrapped AMB, DF, or radiolabel by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), and concentrated by using 300 kDa Microsep filters (Filtron, Breda, The Netherlands). Phospholipid concentration was determined by a phosphate assay [22]. AMB concentration was determined spectrophotometrically at 405 nm, after destruction of the liposomes in DMSO/methanol (1:1, v/v).

### 2.3. Determination of maximum tolerated dosage (MTD)

Toxicity of AMB-DOC, AMB-LIP, PEG-AMB-LIP type 1, and PEG-AMB-LIP type 2 was measured in uninfected specific pathogen free female BALB/c mice (14-to 20-week-old, 22–27 g Iffa Credo, L'Arbresle, France). Mice (6 per group) were treated i.v. for 5 consecutive days with each of the preparations once daily (q 24 h) with AMB dosages ranging from 0.1 to 25 mg/kg per day. Acute mortality was assessed directly following injection of the preparation. Blood urea nitrogen (BUN) and serum creatinine (CREAT), as parameters for renal toxicity, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), as parameters for liver toxicity, were determined by established methods in serum samples of mice killed at 24 h after termination of treatment. The MTD was defined as the maximum dosage that did not result in death during treatment or more than 3-fold increase in the indices for renal and liver function, as compared to untreated mice.

### 2.4. Biodistribution studies

Biodistribution of AMB-LIP, PLACEBO-LIP, PEG-AMB-LIP type 2 and PEG-PLACEBO-LIP type 2 was investigated. Radiolabeled liposomes were administered i.v. at 85 μmol lipid/kg (equivalent to 9 mg/kg AMB) as a single dose in uninfected mice. In two separate experiments of three mice for each different time interval after injection, 200 μl blood was collected from the mice in heparinized tubes. Mice were killed, and whole organs (liver, spleen, lungs, and kidneys) were excised. Blood and organs, as well as the injected dosage of liposomes were counted for $^{67}$Ga-DF in a gamma counter (Minaxy 5530, Packard Instruments, Downers Grove, USA). For the AMB containing preparations blood or organs from three mice were pooled and organs were homogenized in distilled water. Blood and tissue samples were stored at −80°C until just before HPLC analysis, as described before [23]. In a separate experiment using $^{111}$In-labeled red blood cells [24] blood volume was determined to be 7.5% of body weight and blood correction factors were determined for each organ.

## 3. Results and discussion

The MTDs in uninfected mice of AMB-DOC, AMB-LIP, PEG-AMB-LIP type 1, and PEG-AMB-LIP type 2 are presented in Table 1. The MTD of AMB-DOC in terms of death during treatment was 0.8 mg/kg per day. At this dosage renal or liver toxicity was not seen. With AMB-LIP (a formulation with a lipid composition as in AmBisome®) the MTD with no death during treatment was 19 mg/kg per day. At this dosage renal toxicity was not yet observed, whereas the MTD with respect to liver toxicity was 11 mg/kg per day. Incorporation of PEG-DSPE resulting in PEG-AMB-LIP type 1 led to a preparation that was as toxic as AMB-DOC. For PEG-AMB-LIP type 2 death during treatment was shown at dosages higher than 13 mg/kg per day. At this dosage renal toxicity was not seen. For liver toxicity the MTD was 9 mg/kg per day. The high

Table 1
Maximum tolerated dosage (MTD) of AMB-DOC, AMB-LIP, PEG-AMB-LIP type 1, and PEG-AMB-LIP type 2 in uninfected mice[a]

| Parameter of toxicity | MTD[b] (mg/kg per day) | | | |
|---|---|---|---|---|
| | AMB-DOC | AMB-LIP | PEG-AMB-LIP type 1 | PEG-AMB-LIP type 2 |
| Death during treatment | 0.8 | 19 | 0.8 | 13 |
| Impaired renal function | >0.8 | >19 | >0.8 | >13 |
| Impaired liver function | >0.8 | 11 | >0.8 | 9 |

[a]Mice were treated i.v. for five consecutive days once daily (q 24 h) with AMB-DOC or with sonicated liposomes with an average diameter of 100 nm: AMB-LIP (HSPC/Chol/DSPG/AMB), PEG-AMB-LIP type 1 (PEG-DSPE/HSPC/Chol/DSPG/AMB), or PEG-AMB-LIP type 2 (PEG-DSPE/HSPC/Chol/AMB). AMB dosages ranged from 0.1 to 1.0 mg/kg per day in steps of 0.1 mg/kg per day, and above 1.0 mg/kg per day in steps of 2.0 mg/kg per day. Two experiments of three mice each ($n=6$) for each dosage were performed.
[b]Toxicity was determined in terms of death during treatment, or more than 3-fold increase in the indices for renal function (BUN and CREAT) and liver function (ASAT, ALAT) as compared to placebo-treated mice, determined at 24 h after termination of treatment.

toxicity of the PEG-AMB-LIP type 1 preparation is probably due to a very toxic subpopulation of small particles containing AMB. A heterogeneity in particle size was observed after ultracentrifugation of the PEG-AMB-LIP type 1 preparation at $260\,000 \times g$. It was shown by dynamic light scattering measurements that particles of approximately 100 nm containing AMB were sedimented at this speed, whereas small particles of approximately 40 nm containing AMB remained in the supernatant. There was a marked difference with respect to toxicity in terms of acute death in mice between the particles of 40 and 100 nm. Acute death was observed after i.v. administration of the 40 nm AMB-particles at a dosage of 0.6 mg/kg, whereas no toxicity was observed with the 100 nm AMB-particles at a dosage of 6 mg/kg. As a first step in the preparation of PEG-AMB-LIP type 1, AMB is complexed to DSPG. This lipophilic AMB-DSPG complex is inserted in the phospholipid bilayer during spray-drying and thus helps to retain AMB in the liposome. PEG-DSPE is added to this formulation in which AMB is already complexed to DSPG. It is conceivable that competition between PEG-DSPE and DSPG for complexation of AMB occurred, resulting in the formation of highly toxic AMB-containing lipid particles of 40 nm beside less toxic 100 nm AMB-liposomes. In PEG-AMB-LIP type 2 in which DSPG was omitted from the preparation, AMB was complexed to PEG-DSPE. PEG-AMB-LIP type 2 did not show the heterogeneity in particle size, and this was also not observed for AMB-LIP. As the PEG-AMB-LIP type 1 preparation was very toxic, these liposomes were omitted from further studies on biodistribution that were performed with AMB-LIP and PEG-AMB-LIP type 2.

Biodistribution studies were performed with $^{67}$Ga-DF labeled liposomes. $^{67}$Ga-DF was used as marker for intact liposomes. In studies with AMB-containing liposomes both $^{67}$Ga-DF levels as well as AMB concentrations were determined. For both AMB-LIP as well as PEG-AMB-LIP type 2, AMB levels as percentage of injected dosage in blood and liver + spleen were equivalent to levels of intact liposomes (Fig. 1 and Fig. 2), suggesting that AMB levels primarily represent liposome-associated AMB. For both AMB-LIP and PEG-AMB-LIP type 2 levels in kidney and lung never exceeded 3% and 1% of injected liposome dose, respectively (data not shown). Comparison of the blood residence of AMB-LIP versus PLACEBO-LIP shows that the presence of AMB in the liposome preparation induced a dramatic drop in blood level of intact liposomes within 5 min after administration (Fig. 1). As this initial drop is seen in both the $^{67}$Ga-DF marker as well as in AMB concentration (Fig. 1) it is unlikely that this initial decrease is due to leakage of $^{67}$Ga-DF or AMB. Although the initial hepatosplenic uptake of AMB-LIP within 5 min after administration is high (29% of injected dosage), this can still not account for the low blood level (47% of injected dosage) at this time point. As the exact localization of the distributed liposomes in other organs, such as bone marrow, brain, muscle, and skin at 5 min after administration was not determined, the rapid elimination of AMB-LIP is unexplained. After this initial drop in blood level of liposomal AMB a relatively prolonged blood residence

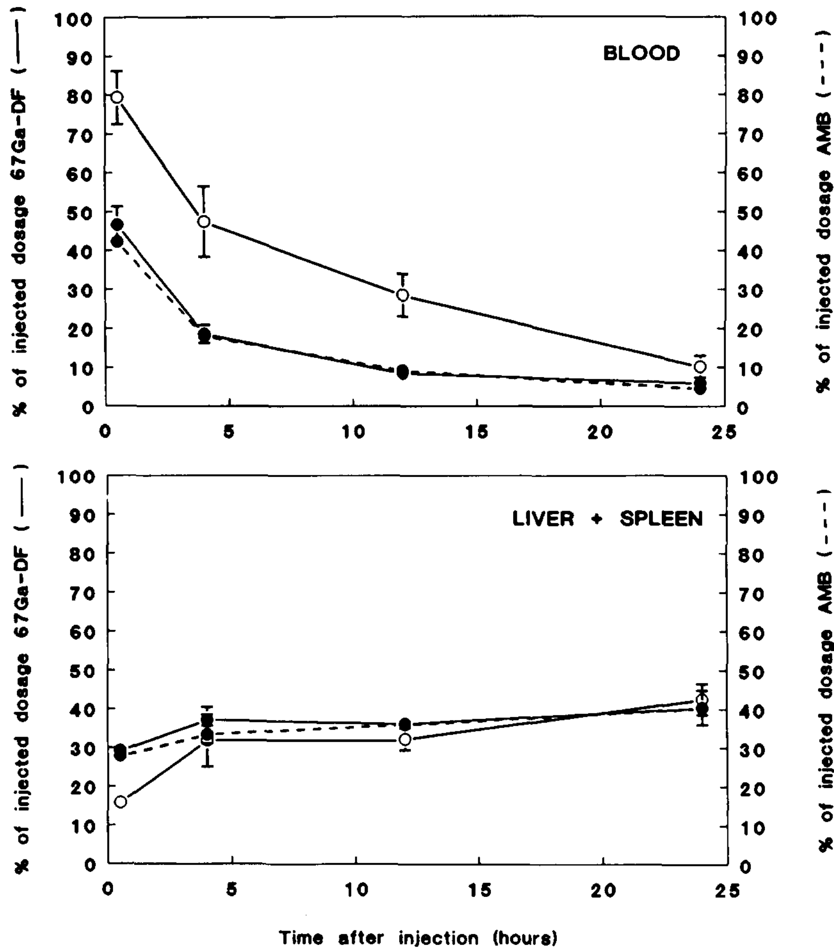


Fig. 1. Biodistribution of $^{67}$Ga-DF labeled liposomes at various intervals after i.v. injection. Sonicated liposomes with an average diameter of 100 nm were injected intravenously in mice at a dosage of 85 $\mu$mol lipid/kg. (○) PLACEBO-LIP: HSPC/Chol/DSPG; (●) AMB-LIP: HSPC/Chol/DSPG/AMB. Data are expressed as percentage of injected dosage cpm $^{67}$Ga-DF (solid line) as mean ± S.D. for two separate experiments of three mice each ($n = 6$), or as percentage of injected dosage AMB (broken line) as mean ± range for two separate experiments with in each experiment blood or tissue from three mice pooled. Where no error-bars are seen the range is smaller than the data points.

time was observed with 6% of injected dosage circulating at 24 h after administration. In line with expectations, it was shown that inclusion of PEG-DSPE into PEG-AMB-LIP type 2 resulted in a prolonged blood residence time of liposomal AMB as compared to AMB-LIP. Unfortunately, due to problems with respect to aggregation of liposomes during preparation, it was not possible to compare PEG-AMB-LIP type 2 with its appropriate control, i.e. non-pegylated AMB-liposomes with omission of not only PEG-DSPE but also DSPG. Successful liposomal encapsulation of AMB requires that AMB is complexed to either DSPG, as in AMB-LIP, or to PEG-DSPE, as in PEG-AMB-LIP type 2. An effect of AMB on blood residence time of the pegylated liposomes was not observed with PEG-AMB-LIP type 2 (Fig. 2). Both PEG-PLACEBO-LIP type 2 as well as PEG-AMB-LIP type 2 showed a long blood residence time with 20–30% of the injected dosage of liposomes still circulating at 24 h after administration. These results are in good agreement with those reported elsewhere for PEG-liposomes with similar particle size [6–8,18]. However, in the present study a relatively high hepatosplenic uptake of the liposomes of 34–43% was observed at 24 h after administration, as compared to the other studies [6–8,18] in which the hepatosplenic uptake ranged from 7 to 28%

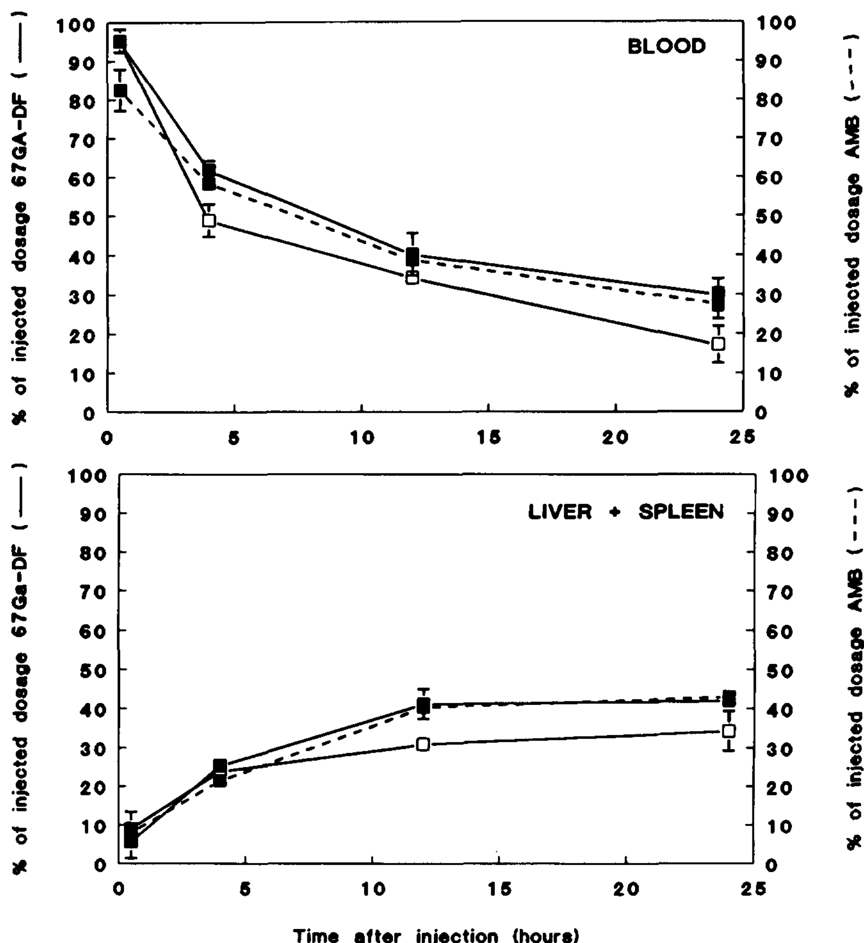


Fig. 2. Biodistribution of $^{67}$Ga-DF labeled liposomes at various intervals after i.v. injection. Sonicated liposomes with an average diameter of 100 nm were injected intravenously in mice at a dosage of 85 $\mu$mol lipid/kg. (□) PEG-PLACEBO-LIP type 2: PEG-DSPE/HSPC/Chol; (■) PEG-AMB-LIP type 2: PEG-DSPE/HSPC/Chol/AMB. Data are expressed as percentage of injected dosage cpm $^{67}$Ga-DF (solid line) as mean ± S.D. for two separate experiments of three mice each ($n = 6$), or as percentage of injected dosage AMB (broken line) as mean ± range for two separate experiments with in each experiment blood or tissue from three mice pooled. Where no error-bars are seen the range is smaller than the data points.

at 24 h after i.v. administration. This might be related to differences in size distribution of the liposomes dispersion, as different methods for liposome sizing were used (sonication in the present study versus extrusion in the other studies). It is possible that sonication, as compared to the extrusion method, yields a preparation with relatively increased numbers of liposomes smaller than 100 nm. Extravascular localization of these small liposomes after passage through the fenestrated vascular endothelial lining of the liver sinusoids may occur. In conclusion, incorporation of PEG-DSPE in AMB-LIP with AMB complexed to DSPG (PEG-AMB-LIP type 1) results in an extremely toxic preparation. When AMB is complexed to PEG-DSPE and DSPG is omitted from the preparation (PEG-AMB-LIP type 2) toxicity is substantially reduced. In comparison with AMB-LIP this PEG-AMB-LIP type 2 preparation shows a slightly increased toxicity. Blood residence time of liposomal AMB is prolonged with PEG-AMB-LIP type 2 as compared to AMB-LIP. To what extent the increased toxicity as well as prolonged blood residence time of liposomal AMB with PEG-AMB-LIP type 2 are determining factors with respect to improved efficacy as compared to AMB-LIP in the

treatment of fungal infections is now under investigation.

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