J. Pharm. Pharmacol. 1987, 39: 24–28
Received April 21, 1986

In-vivo studies of amphotericin B liposomes derived from proliposomes: effect of formulation on toxicity and tissue disposition of the drug in mice

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The repeat dose toxicity of various liposomal formulations containing amphotericin B has been determined in mice. In general, small liposomes (e.g. 100–150 nm) were found to be more toxic than their larger counterparts (e.g. about 2000 nm). However, the repeat dose toxicity of small liposomes could be diminished substantially by the inclusion of sterol (i.e. ergosterol) into the liposomal membranes. Tissue accumulation studies of amphotericin B after repeat dosing may be a useful adjunct to formulation development.

One of the principal virtues of liposomal drug delivery systems is their flexibility, for example, changes in particle size, surface charge and lipid composition can markedly affect the pharmacokinetics and tissue distribution of drugs in-vivo.

In general, liposomes injected intravenously are removed by cells of the reticuloendothelial system. Interaction with these cells has been shown to be influenced by liposome charge, for example. Thus, while Raz et al (1981) found that negatively charged liposomes were phagocytosed at a significantly faster rate than neutral liposomes, Schwendener et al (1984) found that liposome interaction with macrophages was increased with increasing strength of liposomal positive charge. Modifications to liposome composition can affect their uptake by macrophages. For example, inclusion of high levels of cholesterol into vesicle membranes has been shown to reduce their uptake by macrophages (Johnson 1975). While uptake of liposomes composed of phosphatidylcholine/phosphatidylserine by the liver was found to be higher than for liposomes composed of distearoylphosphatidylcholine/cholesterol, the converse situation was found for the spleen (Gotfredson et al 1983).

Within the liver, liposome particle size has been shown to influence the pattern of their uptake by both parenchymal and Kupffer cells. Thus, large liposomes (about 0·5 μm) were taken up rapidly, mainly by Kupffer cells, while small liposomes (about 0·08 μm) were taken up only slowly by this cell type; for parenchymal cells however, the converse was found (Rahman et al 1982). Support for these observations by Roerdink et al (1984) has also shown that release of liposomal degradation products occurred principally from Kupffer rather than parenchymal cells.

Thus, it is conceivable that formulation modifications to liposomes such as size, charge and composition may not only affect the sites of drug disposition but also the manner in which the deposited material is degraded/eliminated which may subsequently affect the toxicity of the product. In this study, we have examined the effect of administering various liposome formulations containing amphotericin B to mice. Liposome encapsulation of amphotericin B has been used by many workers to reduce toxicity associated with free drug. Thus, liposomal amphotericin B has been shown to be efficacious against candidiasis (Lopez-Berestein et al 1983a; Ahrens et al 1984; Tremblay et al 1984), murine cryptococcosis (Graybill et al 1982), histoplasmosis (Taylor et al 1982) and experimental leishmaniasis (New et al 1981; Panosian et al 1984). The liposome formulations have been diverse. Hopfer et al (1984) found that the inclusion of cholesterol or ergosterol into amphotericin B-containing liposomes caused a significant increase in the minimal fungicidal concentration of drug against various yeast isolates. On the other hand, Lopez-Berestein et al (1983b) found no significant difference in the survival time of mice infected with *Candida albicans* when they were treated with vesicles containing both amphotericin B and sterol, versus sterol-free vesicles.

The present study has investigated how both liposome size and composition can affect chronic toxicity and how this effect may be modified by the inclusion of sterol (i.e. ergosterol) into a given formulation.

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MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (sodium salt; DMPG) were from Avanti Polar Lipids, Birmingham, Alabama, USA, egg lecithin (EL) from Lipoid KG, Germany, ergosterol (Erg) from Sigma, Poole, Dorset, UK and amphotericin B (AmB) was from E. R. Squibb and Sons Inc., New Jersey, USA. All other materials were of analytical reagent grade.

With one exception, all the liposome formulations were derived from proliposomes (prepared on either sorbitol or sodium chloride as carrier). Proliposomes are a dry granular product composed of a water-soluble carrier material coated with dry liposomal components (e.g. phospholipids, sterol, drug). Addition of water above the phase transition temperature of the component lipids results in the formation of an isotonic liposomal suspension. Extensive details of their preparation and characterization are to be found elsewhere (Payne et al 1986a, b). Brief details of proliposome preparation are outlined below.

Proliposome manufacture

Sorbitol (10 g) or sodium chloride (1·8 g) was placed in a round-bottomed flask which was subsequently attached to a rotary evaporator (e.g. Büchi Rotavapor-R). With moderate heating of the carrier material (35–45 °C), aliquots of a methanolic (or methanol/chloroform) solution of phospholipid/sterol/amphotericin B were added sequentially under vacuum until the ratio of liposomal components to carrier material was 1 : 4·13 (sorbitol) or 5·4 : 1 (sodium chloride). The precise formulations are detailed below. Resultant proliposomes were sieved to remove material < 75 and > 600 μm in diameter.

Cast film liposome manufacture

Cast film liposomes were prepared by evaporating a lipid/drug solution to dryness in a round-bottomed flask, and hydrating the film with aqueous medium.

Proliposome formulations

All proliposome formulations were prepared in a similar manner. The composition of each formulation is described in Table 1.

A lipid to amphotericin B weight ratio of 18 : 1 was maintained throughout except for Formulation 6 where a ratio of 24·2 : 1 was used. These ratios were chosen to avoid the presence of extra-liposomal amphotericin B. Approximately 100% encapsulation efficiency was achieved in all formulations.

Table 1. LD50 values in mice for liposomes containing amphotericin B.

Formulation No.	Liposome type	Molar ratio	Preparation (and carrier)	Size (μm)	LD50 (mg kg^{-1})
1	DMPC/DMPG	7:3	Cast film	2·0	11·7
2	DMPC/DMPG	7:3	Proliposome (Sorbitol)	0·13	13·0
3	DMPC/DMPG/Erg	7:3:0·46	Proliposome (Sorbitol)	0·12	>18·8
4	DMPC/DMPG	7:3	Proliposome (Sodium chloride)	1·7	N.D.
5	DMPC/DMPG	50·1	Proliposome (Sorbitol)	3·0	N.D.
6	EL/Erg	12·2:1	Proliposome (Sorbitol)	2·5	>25·0

N.D. = Not determined.

Formulation 6 was hydrated with water at 20–25 °C whereas all other formulations were hydrated with water at 37 °C (or 0·9% w/v sodium chloride at 37 °C for Formulation 1).

Dosing of mice

Groups of ten male mice (20–25 g) (Hacking and Churchill Strain CFLP) were injected with up to 0·4 mL liposome suspension via the tail vein. For repeat dose studies, groups of 10 mice were injected at 24-hourly intervals (dose as indicated in relevant Table) for three consecutive days.

Biodistribution studies

For biodistribution studies, kidneys, liver, lungs and spleen were removed at the completion of a repeat dose study (i.e. 18 h after the last injection), weighed and analysed for amphotericin B content by HPLC.

Measurement of LD50 (chronic)

Groups of ten mice were injected intravenously with liposomal amphotericin B in the range amphotericin B 5–25 mg kg^{-1}. Deaths were recorded over 5–10 days, most occurring within 2 days.

Preparation of tissue samples for amphotericin B analysis

Samples of tissue (0·15 g–0·2 g) were weighed accurately into a tissue grinder and 1 mL DMSO–methanol–water (10 : 9 : 1) added. After grinding for 5 min, the contents were transferred to a 10 mL volumetric flask and made up to volume with DMSO–methanol–water (10 : 9 : 1). The suspension was clarified by centrifugation and the supernatant subjected to HPLC analysis. Mean recoveries (n = 3) were found to be: lung (AmB 100 μg g^{-1}) = 92·3%; spleen (AmB 50 μg g^{-1}) = 94·9%; kidney (AmB 10 μg g^{-1}) = 93·5%; liver (AmB 120 μg g^{-1}) =

93·3%. Tissue containing no drug gave no response at the retention time for amphotericin B.

HPLC analysis of amphotericin B

Acetate buffer (pH 4·12) contained 100 mL sodium acetate solution (3·86% w/v) and 8 mL glacial acetic acid diluted to 1 L with water.

HPLC mobile phase contained acetate buffer–acetonitrile–methanol (1·1 : 1·4 : 0·5). Flow rate = 1 mL min^{-1}. Detector wavelength = 406 nm. Column = MicroBondapak C18 (Waters) 3·9 mm × 30 cm (fitted with guard column).

Particle size analysis

Analysis of liposome suspensions was performed using either a Coulter TAII particle sizer or a Malvern 4600M photon correlation spectrometer as described extensively elsewhere (Payne et al 1986b).

RESULTS AND DISCUSSION

To assess the relative toxicity of the different formulations, LD50 determinations were performed initially. LD50 data presented in Table 1 indicate that liposomes composed of egg lecithin/ergosterol/AmB were relatively non-toxic (LD50 greater than 25 mg kg^{-1}). In contrast, administration of large DMPC/DMPG/AmB liposomes derived from cast films (mass median volume equivalent diameter = 2·0 μm) resulted in comparatively lower LD50 values (11·7 mg kg^{-1}) similar to those obtained with small DMPC/DMPG/AmB liposomes (mean = 0·13 μm: LD50 = 13·0 mg kg^{-1}). Inclusion of ergosterol into the latter formulation resulted in an increase in LD50 value to greater than 18·8 mg kg^{-1} (an accurate value was not obtained due to physical limitations in injection volume). In comparison, Fungizone (amphotericin B desoxycholate) had an LD50 value of about 1 mg kg^{-1}. Thus, on the basis of LD50 data, all the liposomal formulations tested were less toxic than the product currently available.

Table 2. Effect of repeat dosing of amphotericin B liposomes in mice.

Formulation No. (Table 1)	Dose (mg kg^{-1})	Frequency (Days)	No. deaths after 5 days (maximum = 10 mice)
1	10·0	3	0
2	10·0	3	6
3	12·1	3	0
5	11·9	3	9
6	12·1	3	0

Repeat dose studies did not correlate entirely with trends indicated by LD50 data. Results in Table 2 indicate that repeat dosing with small DMPC/DMPG liposomes (Formulation 2) resulted in greater toxicity than their larger DMPC/DMPG counterparts (Formulation 1) (6/10 versus 0/10 deaths, respectively) despite similar LD50 values.

Liposomes composed of egg lecithin/ergosterol/AmB were essentially non-toxic in this system while inclusion of ergosterol into the membranes of small DMPC/DMPG/AmB liposomes eliminated toxicity normally associated with repeat dosing. Thus, small liposomes appeared to be more toxic on repeat dosing than their larger counterparts unless ergosterol was present in the formulation. The observed reduction in toxicity associated with ergosterol inclusion may be the result of complex formation between ergosterol and amphotericin B (Readio & Bittman 1982; Vertut-Croquin et al 1983) preventing toxicity normally associated with free drug.

However, liposome size itself was not the sole factor associated with toxicity. Thus, amphotericin B liposomes composed of a 50 : 1 molar ratio of DMPC : DMPG (mass median volume equivalent diameter = 3·0 μm), although slightly larger than both cast film liposomes and liposomes derived from egg lecithin/ergosterol/AmB proliposomes, were found to be toxic in mice (Table 2). Therefore lipid composition may effect more subtle interactions in-vivo despite the observation that all the lipids used in these studies had LD50 values in mice in excess of 2000 mg kg^{-1}.

It is possible that whilst the toxicity of some of the formulations has been reduced by the inclusion of ergosterol, their efficacy has also been compromised. Hopfer et al (1984) demonstrated that liposome formulations containing ergosterol can increase the maximum fungicidal concentration against yeast isolates more than four-fold when compared with Fungizone. However, our experience with the EL/Erg/AmB formulation in an in-vivo murine candidiasis model would suggest comparable efficacy with Fungizone (D. P. Bonner, personal communication).

Drug disposition profiles in various tissues following repeat dosing of amphotericin B liposomes are summarized in Table 3. Whilst reasonably consistent levels of amphotericin B in kidneys were found for all four formulations tested, administration of EL/Erg/AmB liposomes resulted in very high levels of drug in the lung. This may be the result of capillary occlusion in this organ, a feature consistent with the rapid removal of drug from the circulation following i.v.

injection (R.F. Cosgrove, personal communication). However, the 50:1 DMPC:DMPG formulation in this study was of similar mean size but resulted in significantly lower levels of amphotericin B in lung tissue. Thus, the nature of the lipid composition in the formulation appeared to modify site specificity. Liver levels of amphotericin B were reasonably consistent for all formulations except for liposomes derived from cast films. Splenic drug levels ranged from 83·6 μg g^{-1} for liposomes derived from cast films to 644·5 μg g^{-1} for 50:1 DMPC:DMPG liposomes.

Table 3. Tissue concentrations of amphotericin B following repeat dosing of mice with amphotericin B liposomes.

	Egg lecithin : ergosterol (12·2 : 1)		DMPC : DMPG (7 : 3) Cast Film		DMPC : DMPG (7 : 3) Proliposome (sodium chloride)		DMPC : DMPG (50 : 1) Proliposome (sorbitol)	
Tissue	μg g^{-1}	μg g^{-1} organ	μg g^{-1}	μg g^{-1} organ	μg g^{-1}	μg g^{-1} organ	μg g^{-1}	μg g^{-1} organ
Liver	124·4	16·0	29·3	6·3	90·0	16·9	121·4	46·0
Lung	582·1	97·5	154·4	29·2	11·1	1·6	14·7	2·2
Kidney	12·4	3·2	10·3	3·2	14·8	4·8	12·8	5·2
Spleen	201·8	27·0	83·6	13·7	150·8	21·0	644·5	51·6

Whilst no attempt has been made to perform a mass balance in this series of experiments, recoveries of amphotericin B from tissues at the completion of repeat dose studies varied considerably. After the administration of three injections (a total of 900 μg amphotericin B per mouse), recoveries from liver, spleen, lungs and kidneys varied from 43 to 144 μg amphotericin B per mouse (depending upon liposome formulation) (see Table 3). Studies in dogs (Craven et al 1979) have suggested that biliary excretion of amphotericin B is only of modest importance regarding elimination of the drug. Excretion of amphotericin B in the urine was found to be prolonged (23–35 days following administration of only a single dose of drug). However, these studies failed to account for the remaining 60–70% of the administered dose.

Since comparable studies have not been performed in mice, it is not possible to draw firm conclusions regarding the amphotericin B not accounted for by disposition in liver, spleen, kidneys and lungs. Whilst we have yet to establish a causal relationship between tissue drug concentrations and relative liposome toxicity, it is conceivable that monitoring of these parameters could give an indication of potentially toxic formulations during their early developmental stages.

Conclusions

In conclusion, the optimization of liposome formulations is necessarily a compromise between many, often conflicting factors. Whilst all the formulations examined in this study were substantially less toxic than Fungizone, other factors such as phospholipid type/charge, presence/absence of ergosterol and liposome size all exerted profound effects on the relative toxicities of the final liposome formulations. As an aid to evaluating potential formulations, we have devised a repeat dose study to distinguish between toxic/non-toxic formulations and have shown that tissue drug profiles may be a useful adjunct to formulation optimization.

Acknowledgement

We would like to thank Miss Sandra Baines for her expert technical assistance.

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