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Amphotericin B–phospholipid interactions responsible for reduced mammalian cell toxicity

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When interacting with phospholipid in an aqueous environment, amphotericin B forms unusual structures of markedly reduced toxicity (Janoff et al. (1988) Proc. Natl. Acad. Sci. USA 85, 6122–6126). These structures, which appear ribbon-like by freeze-fracture electron microscopy (EM), are found exclusively at amphotericin B to lipid mole ratios of 1:3 to 1:1. At lower mole ratios they occur in combination with liposomes. Circular dichroism (CD) spectra revealed two distinct modes of lipid–amphotericin B interaction, one for liposomes and one for the ribbon-like structures. In isolated liposomes, amphotericin B which comprised 3–4 mole percent of the bulk lipid was monomeric and exhibited a hemolytic activity comparable to amphotericin B suspended in deoxycholate. Above 3–4 mole percent amphotericin B, ribbon-like structures emerged and CD spectra indicated drug-lipid complexation. Minimal inhibitory concentrations for *Candida albicans* of liposomal and complexed amphotericin B were comparable and could be attributed to amphotericin a release as a result of lipid breakdown within the ribbon-like material by a heat labile extracellular yeast product (lipase). Negative stain EM of the ribbon-like structures indicated that the ribbon-like appearance seen by freeze-fracture EM arises as a consequence of the cross-fracturing of what are aggregated, collapsed single lamellar, presumably interdigitated, membranes. Studies examining complexation of amphotericin B with either DMPC or DMPG demonstrated that headgroup interactions played little role in the formation of the ribbon-like structures. With these results we propose that ribbon-like structures result from phase separation of amphotericin B-phospholipid complexes within the phospholipid matrix such that amphotericin B release, and thus acute toxicity, is curtailed. Formation of amphotericin B-lipid structures such as those described here indicates a possible new role for lipid as a stabilizing matrix for drug delivery of lipophilic substances, specifically where a highly ordered packing arrangement between lipid and compound can be achieved.

Introduction

The ability of amphotericin B, an antifungal drug, to deplete transmembrane ion gradients has been studied extensively for over two decades [1–5]. Presumably, the preferential destruction of fungal cells mediated by this drug arises from its greater binding affinity to ergosterol, the predominant sterol in fungal cell membranes, compared to cholesterol, the predominant sterol in mammalian cell membranes [6,7].

Although the drug of choice for treatment of systemic fungal infections, amphotericin B in its current deoxycholate formulation still remains significantly toxic to its mammalian host [8]. One approach to this problem has involved liposomal delivery systems. Lopez-Berestein et al. [9] and others (reviewed by Bratjburg et al. [10]) have shown that incorporating amphotericin B at 5–10 mole percent in liposome suspensions lowers its toxicity from an LD_{50} of 1 mg/kg to 12 mg/kg in mice with little compromise to efficacy.

Recently, we have shown that incorporation of amphotericin B into lipid systems at much higher mole percentages (25 and 50 mole percent) results in a significantly enhanced attenuation of mammalian cell

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DHPC, dihexadecylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine: CD, circular dichroism; LD_{50}, dosage at which 50% of the mice expire (time course of 10 days); RBC, red blood cell; MIC, minimal inhibitory concentration; TLC, thin-layer chromatography; ABLC, amphotericin B-lipid complex.

but not yeast cell toxicity. LD_{50} values of 20 mg/kg or higher in mice could be achieved depending on formulation protocols [11,12]. Freeze-fracture electron microscopy of these emulsions revealed unusual structures that we termed 'ribbon-like'. Amphotericin B in this form (amphotericin B-lipid complex, ABLC) has subsequently been shown to alleviate acute toxicity without compromising efficacy in human clinical trials [13–19]. Since we also found 'ribbons' mixed with typical liposome structures in 5 mole percent amphotericin B preparations, we suggested that the previously reported reductions in toxicity noted in 'liposomal' formulations [9,20] might actually be attributed to these unusual high-mole percent amphotericin B-lipid structures.

Although we could systematically relate decreases in amphotericin B toxicity to formation of high mole percent amphotericin B-lipid 'ribbon' complexes, the molecular basis for the toxicity reduction remained unclear. Here we examined amphotericin B-lipid mixtures and their subpopulations to delineate the lipid–amphotericin B interactions that influence toxicity. Our results indicate that two distinct associations occur between lipid and amphotericin B and from this we have proposed a model for the molecular basis of the ribbon-like structures.

Materials and Methods

Chemicals. Amphotericin B was obtained from Bristol Myers-Squibb (New Brunswick, NJ). Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL). [^{14}C]DMPC was obtained from Amersham Corporation (Arlington Heights, IL). Fungizone ® was obtained from GIBCO Laboratories (Grand Island, NY). Sabouraud dextrose broth (BBL ®) was purchased from Becton Dickinson Microbiology Systems (Cockeysville, MD).

Amphotericin B-lipid preparations. Amphotericin B was dissolved at 0.1 mg/ml in methanol and added to a round bottom flask containing the appropriate amount of lipid already dried to a thin film from chloroform. The methanol was removed by vacuum rotary evaporation and the resultant amphotericin B-lipid film was resuspended at 45°C in an aqueous solution of 20 mM Hepes and 150 mM NaCl (pH 7.4). This buffer was used throughout. The suspension was then bath sonicated for 30 min at 5–20°C in order to reduce particle size for in vivo LD_{50} testing. Analysis of sonicated and unsonicated preparations revealed that sonication did not enhance formation of the 'unusual' amphotericin B ribbon-like structures within our preparations of low mole percentage amphotericin B, as suggested by Grant et al. [21], (data not shown).

Lipid and amphotericin B assays. Phospholipid content was determined according to the procedure of Chen et al. [22]. For amphotericin a, aliquots of each drug-lipid preparation were diluted to 5–8 μM in methanol (monomeric) and the absorbance at 405 nm was recorded using a Shimadzu UV-160 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Concentrations were established from comparison to standard curves.

Density gradient centrifugation. Typically, 0.2 ml of a 2 mg/ml amphotericin B sample was placed on 5 ml of a 0–40% (w/v) sucrose gradient (in 20 mM Hepes, 150 mM NaCl, pH 7.4) and centrifuged at 230 000 × g for 22 h at 18–20°C. In the case of Fig. 1, the gradient ranged from 0 to 30% (w/v) sucrose. The gradients were fractionated from the top into 0.2-ml aliquots which were then assayed for lipid and amphotericin B.

In vitro toxicity. Red blood cell (RBC) hemolysis was used to assess the in vitro toxicity of the various preparations. 0.5 ml of sample was mixed with 0.5 ml of a washed 4% (v/v) RBC suspension in phosphate-buffered saline and the mixture incubated with constant agitation for 20 h at 37°C. Following low-speed centrifugation (2000 rpm for 10 min) 200 μl were taken from above the RBC pellet and diluted with 1 ml of buffer solution. Percent hemolysis was based on the absorbance at 550 nm with 100% hemolysis being the value obtained from a 1:1 dilution of the 4% RBC solution with distilled water.

In vitro efficacy. For minimal inhibitory concentration (MIC), Fungizone ® and drug-lipid complexes were diluted directly in sabouraud dextrose broth. The MIC of the various preparations was determined in microtiter plates against *Candida albicans* (ATCC No. 24433) and *Saccharomyces cerevisiae* (ATCC No. 9763) after 18 h at 30°C as described by McGinnis and Rinaldi [23].

Incubation of amphotericin B-lipid complex with yeast broth. Amphotericin B/lipid mixtures were incubated in sabouraud dextrose broth (BBL ®) that had either never been exposed to yeast or had been used to support yeast (*Candida albicans*) growth for 24 h but had subsequently been separated from yeast cells by low-speed centrifugation followed by filtration through a 0.8 μm polycarbonate filter. 1-ml aliquots of 10 mg/ml amphotericin B at 50 mole percent in DMPC/DMPG (7:3) were added to: (1) 49 ml of broth formerly used to support yeast growth, (2) 49 ml of broth never exposed to yeast, and (3) 49 ml of broth that, following separation from the yeast cells, had been heated to 95°C for 1 h. These mixtures were incubated for 24 h at 37°C with constant stirring in the dark. Absorbance of aliquots that were taken at the beginning and end of the incubation period and dissolved in methanol were identical, suggesting no chemical breakdown of amphotericin B. Amphotericin B-

lipid complex was retrieved from broth by centrifugation (14000 × g). Although at this centrifugal force all of the highly dense amphotericin B-lipid complex should have pelleted, we could not be certain that trace levels of amphotericin B (especially monomeric amphotericin B that may have been released from the complex) were not lost to the broth. The supernatant was removed and the pellet was resuspended in the same Hepes buffer solution described above. This washing procedure was repeated once more with the final pellet resuspended in approx. 2 ml of buffer. Red blood cell hemolysis was assayed as described above. As a control, an aliquot of the drug-lipid suspension that was never exposed to broth was also examined.

Thin-layer chromatography (TLC) of lipids exposed to yeast incubation broth. Following 24 h of incubation at 37°C, broth mixtures containing drug-lipid complexes that were prepared as described above as well as amphotericin B-free DMPC/DMPG liposomes were subjected to lipid extraction [24] and examination by TLC. 1-ml of 10 mg/ml amphotericin B at 50 mole percent in [^{14}C]-DMPC/DMPG (1 μCi/ml) or liposomes without amphotericin B at equivalent lipid concentrations was added to 49 ml of broth that met the conditions of (1), (2) and (3) stated above. Additionally, (4) 1 ml of the drug-lipid preparation was added to 49 ml of Hepes buffer solution which was incubated at 37°C alongside the other samples. Following incubation, 25 ml was taken from each of the eight mixtures and combined with 62.5 ml of methanol and 32 ml of chloroform. The resulting monophase formed two phases upon addition of 32 ml each of water and chloroform. The lower chloroform phase was removed and dried by vacuum rotary evaporation to a final volume of 5 ml. 1 ml of this was then further concentrated (N_2 stream) and applied to a Whatman $^{®}$ silica gel 60 TLC plate (20 × 20 cm) (Baxter Scientific Products, Edison, NJ). A chloroform/methanol/water (65:25:4, v/v) solvent system was used and the plate was developed with iodine. Spots were scraped and assayed for phosphate and radioactivity.

Circular dichroism spectroscopy. For CD measurements, samples were adjusted to 20 to 40 μM and scanned using a AVIV Model 6205 circular dichroism spectrophotometer (AVIV Associates, Lakewood, NJ). Spectra were recorded at ~22°C with a 1-cm path length cell. In some cases, direct measurement of undiluted samples (2 mM) was achieved using a 0.1-mm path length quartz cell (Wilmad Glass Co., Buena, NJ). Temperature was varied using a circulating water bath interfaced to a jacketed sample compartment.

Electron microscopy. For freeze-fracture replicas, a 0.1–0.3-μl aliquot of sample (without cryoprotectant added) was placed between a pair of Balzer copper support plates (Nashua, NH) and rapidly plunged from 21°C into liquid propane at −190°C. Samples were fractured and replicated in a Balzer BAF 400 freeze-fracture unit at $4 \cdot 10^{-7}$ mbar and at −115°C. Replicas were floated off the support plates in 3 M HNO_3, transferred to distilled water and then to 5% sodium hypochlorite (commercial bleach) for overnight cleaning.

For negative staining, the sample was diluted to approx. 0.5 mg/ml lipid and a drop of this placed on a Formvar-carbon coated grid. After 1 min the bulk of the sample was removed with filter paper. A 2% ammonium molybdate solution was then applied and removed after 30 s with filter paper. Representative pictures were taken using a Philips 300 electron microscope.

Results

We have shown previously that amphotericin B incorporated at 25 or 50 mole percent in DMPC/DMPG (7:3) forms structures of unusual ribbon-like morphology resulting in attenuated mammalian cell toxicities [11,12]. Amphotericin B in this form (amphotericin B-lipid complex, ABLC) has recently shown promise in clinical trials [13–19]. Although less strikingly evident, these ribbon-like structures were also observed within preparations containing 5 mole percent Amphotericin B. Above 50 mole percent amphotericin B structures resembling free amphotericin B predominated [25], concomitant with an increase in toxicity. Fig. 1 shows the bimodal sucrose density gradient profile we have found to be typical of amphotericin B/lipid preparations formulated so that amphotericin B comprises 5 mole percent of the bulk lipid (DMPC/DMPG, 7:3). Above 25 mole percent drug, amphotericin B comigrates with lipid on density gradients as a single band [11,12]. In this paper we explore the nature of these and various other preparations.

Circular dichroism (CD) spectra arising from 5, 25, and 50 mole percent amphotericin B in DMPC/DMPG (7:3) are shown in Fig. 2. All spectra displayed broad positive peaks at 320–330 nm indicative of chromophore complexation [26]. These spectra arose reproducibly from separate 25 and 50 mole percent amphotericin B preparations, but not from preparations containing 5 mole percent amphotericin B where wide variability was evident, presumably a function of sample preparation. Examination of several preparations of 5 mole percent amphotericin B in DMPC/DMPG revealed that, while always resolving into two bands on sucrose density gradients, the distribution of amphotericin B between the two populations varied. This suggested that individual subpopulations in this preparation possessed their own characteristic CD spectra.

Analysis of subpopulations

Individual fractions of 25 and 50 mole percent amphotericin B preparations, separated on sucrose den-

sity gradients, gave CD spectra that were identical in shape (except magnitude) to the unseparated samples (data not shown). These data suggest that only one complexation state exists within each of those formulations. CD spectra arising from individual fractions taken from a sucrose density gradient of a 5 mole percent amphotericin B-lipid preparation (shown in Fig. 1) are illustrated in Fig. 3. Clearly each population possessed its own distinct spectrum. The spectra of the higher density fractions (28–37) exhibited features reminiscent of 25 and 50 mole percent preparations while the spectra arising from the lower density fractions (16–25) resembled that of monomeric amphotericin B [26]. For comparison, the CD spectrum of amphotericin B in methanol (monomeric) is shown in Fig. 3 (offset). Spectra of intermediate fractions (26 and 27), where overlap of the two populations occurred, appeared as composites. The amphotericin B content marking the transition point for this sample was 3.5 mole percent.

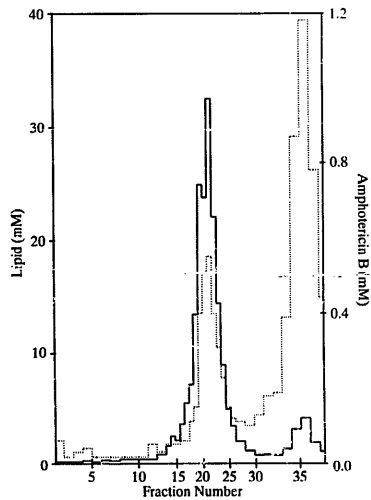


Fig. 1. Sucrose density gradient profile of 5 mole percent amphotericin B in DMPC/DMPG (7:3). 0.5 ml of a 4 mg/ml amphotericin B sample was placed on a 12 ml sucrose gradient consisting of 0–30% sucrose (w/v). Following centrifugation, the gradient was fractionated from the top into 0.4-ml aliquots (0.2-ml aliquots were taken to improve resolution of the liposomal band). Curves represent DMPC/DMPG (———) and amphotericin B (·····) distributions. The peak fractions of the two bands are comprised of 1.7 and 23 mole percent amphotericin B for the low- and high-density populations, respectively. Fractions below number 25 consist of monomeric liposomal associated amphotericin B whereas fractions above number 28 consist of complexed non-liposomal amphotericin B (see text).

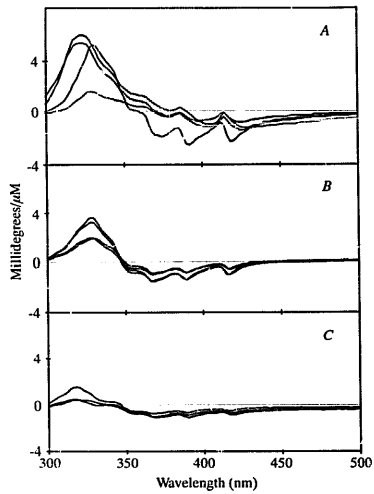


Fig. 2. CD spectra of 20 μM preparations ($n = 4$) of amphotericin B at (A) 5 mole percent, (B) 25 mole percent, and (C) 50 mole percent in DMPC/DMPG (7:3). Although the magnitudes of the peaks and the positions of the broad positive peaks differ for 25 and 50 mole percent polyene preparations, both formulations exhibit four negative peaks at approx. 356, 370, 393 and 422 nm.

The minimum membrane concentration yielding 'complexed' amphotericin B was further examined in unseparated DMPC/DMPG (7:3) preparations formulated with low mole percent amphotericin B (Fig. 4). Because it is a sensitive indicator, the broad positive peak at 330 nm was used to gauge the presence of complexed amphotericin B (Fig. 4 inset). The appearance of complexed material in these samples again occurred between 3 and 4 mole percent amphotericin B. Inspection of density gradient profiles (i.e., Fig. 1) thus revealed that in 5 mole percent drug preparations, amphotericin B is not entirely complexed but is unequally divided between the low-density uncomplexed (monomeric) and high-density complexed forms (20 to 40% as the low-density species). In fact, complete disappearance of monomeric drug does not occur until amphotericin B content reaches 25 mole percent. The inability to detect monomer in the presence of substantial amounts of complexed species (i.e., Fig. 2A) is a consequence of the monomer's much weaker molar ellipticity [26].

Freeze-fracture electron microscopy confirmed that the low-density material containing monomeric amphotericin B was liposomal; liposomes exclusively comprised the lower density material isolated from 5 mole percent preparations (Fig. 5A). The existence of $P_{\beta'}$, or ripple phase, in this material, typical of mixtures of DMPC/DMPG just below the gel to liquid-crystalline phase transition (23°C), indicated that lipid packing was minimally affected by the low level of amphotericin B present. Liposomes found in preparations containing higher amphotericin B mole fractions lack this phase [20,21]. As shown in Fig. 5B, only unusual 'ribbon' structures were found within the high-density complexed fractions.

Negative stain electron microscopy revealed that these high-density structures, seen as ribbon-like in freeze-fracture replicas, arose from aggregated collapsed membranes that were predominantly unilamellar in nature (Fig. 5C). Because the freeze-fracture technique yielded only cross fractures, we speculate that the amphotericin B-lipid complex may not possess the classical bilayer midplane through which a fracture face is revealed, but rather exists as an interdigitated bilayer [27]. Attempts to determine the membrane thickness of amphotericin B-DMPC/DMPG mixtures by small angle X-ray diffraction were unsuccessful as no discernible repeat could be obtained. This result was not surprising, given the unilamellar character observed from negative staining.

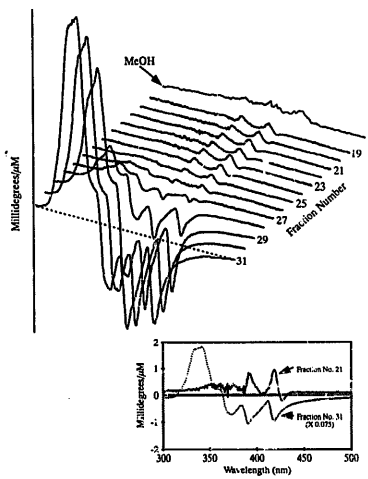


Fig. 3. CD spectra of individual fractions of the 5 mole percent amphotericin B preparation of Fig. 1. Samples were diluted to 10–20 μM amphotericin B and scanned from 500 to 300 nm. For clarity fractions 34–37 were omitted. Shown in the inset are spectra of fractions 21 (——) and 31 (·····). The spectrum of amphotericin B in methanol (20 μM) is shown at the top. Samples were unaffected by sucrose as CD spectra of 5, 25 and 50 mole percent amphotericin B preparations incubated in 40% sucrose were identical to those of the same samples kept in buffer solution. The increase in signal amplitudes evident in fractions containing higher mole percentages amphotericin B is consistent with amphotericin B complexation [26].

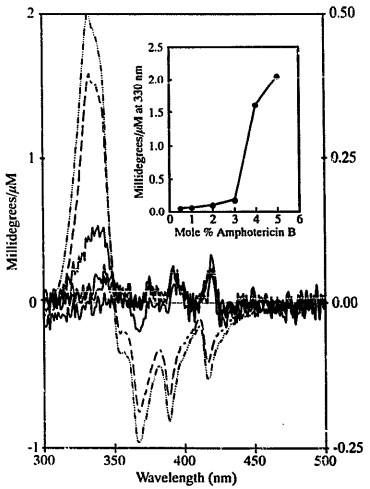
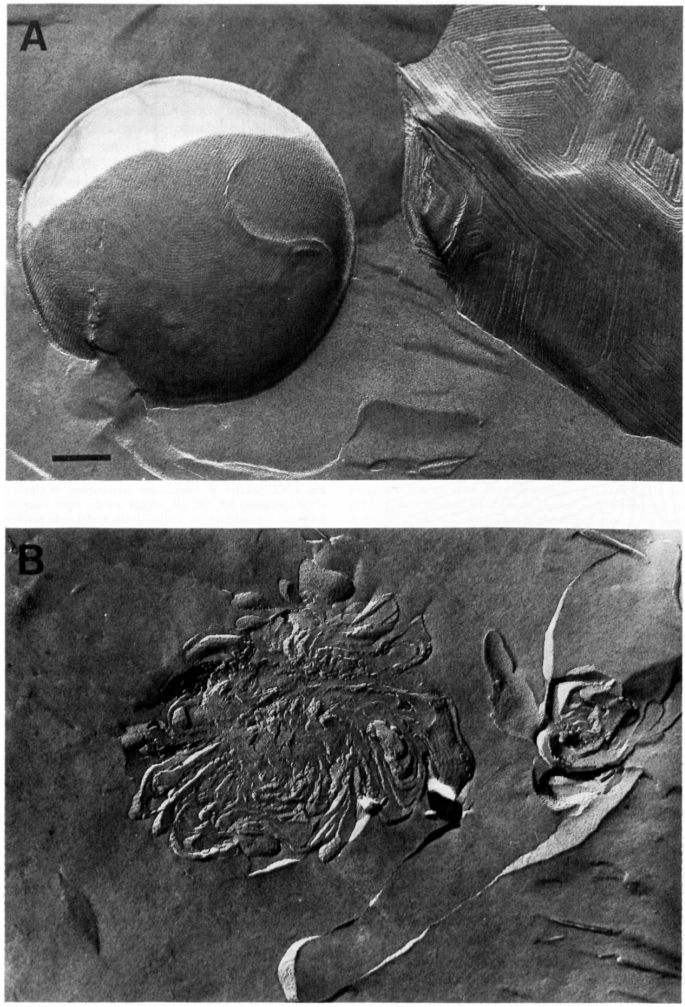


Fig. 4. CD spectra of unseparated preparations of DMPC/DMPG (7:3) containing 0.5, 1.0, 2.0, 3.0 (all use the right y-axis scale) and 4.0 (- - - - -) and 5.0 (–·–·–) (left y-axis scale) mole percent amphotericin B. The major CD peak at 330 nm is plotted versus mole percent amphotericin B (inset). A sudden increase in intensity of this peak occurred between 3 and 4 mole percent drug, which is also the mole percent marking the appearance of complexed species on the density gradient in Fig. 1.

In vitro toxicity and efficacy of the separated populations

As illustrated in Fig. 6, amphotericin B-lipid subpopulations separated from 5 mole percent material differed dramatically in their ability to lyse red blood cells. Material taken from the central fraction of the lower density population was > 1000-times more hemolytic than material taken from the high-density band. In fact, amphotericin B from the low-density


A
B

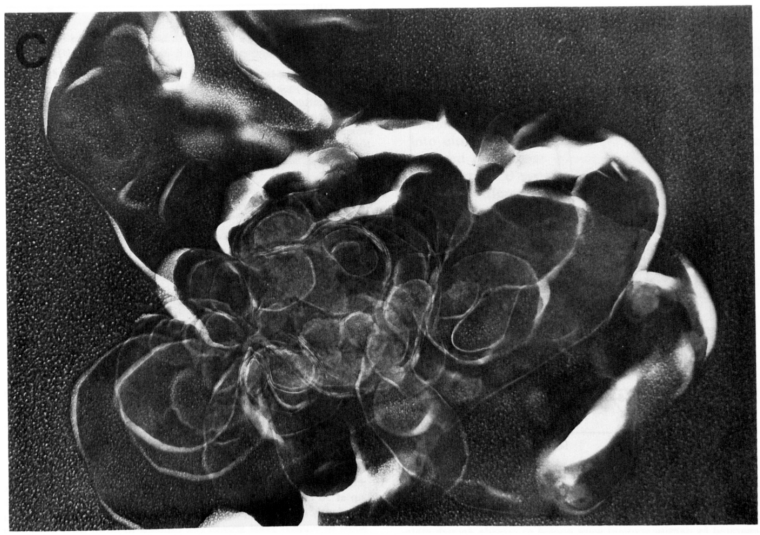


Fig. 5. Freeze-fracture replicas of subpopulations of a 5 mole percent amphotericin B system separated by isopycnic sucrose density centrifugation (see Fig. 1). (A) Fractions taken from the low-density band (1.5 mole percent drug) and (B) fractions taken from the high-density band (25 mole percent drug). Samples were rapidly frozen in liquid propane from −21°C. Micrograph (C) is the high-density material negatively stained. Bar represents 200 nm.

fraction was comparable to a currently used amphotericin B-deoxycholate formulation (Fungizone ®).

The minimal inhibitory concentrations (MICs) against *Candida albicans* and *Saccharomyces cerevisiae* for both the high- and low-density material were equivalent (Table I). Because the two populations differed so dramatically in their ability to lyse red blood cells (RBCs), these experiments identified a significant degree of selective toxicity in the high density material. Such lipid-dependent selective reductions in amphotericin B toxicity have been described previously but mechanistically have been difficult to understand [20,28]. Therefore, we examined the effect of a cell free broth previously used to support yeast growth upon the amphotericin B-lipid complex and its lytic activity (see Materials and Methods for details). Following incubation with isolated yeast broth for 24 h at 37°C, the hemolytic activity of an amphotericin B-lipid sample (formed at approx. 50 mole percent amphotericin B) increased dramatically (Fig. 7). Analysis of this sample revealed a loss of lipid relative to amphotericin B resulting in a system composed of 62 mole percent drug. Amphotericin B-lipid incubated with either broth never exposed to yeast or broth exposed to yeast and subsequently heated to 95°C for 1 h exhibited no hemolytic activity. In these samples drug/lipid ratios remained essentially unchanged (see Fig. 7 legend). The absence of a change in hemolytic activity of the sample incubated with yeast-exposed heat-treated broth suggested that a denaturable protein (e.g., lipase) played a role in 'activating' the complex.

Investigating this further, we used [^{14}C]DMPC (labeled at C-1 on both fatty acyl chains) in the experiment described above but, following incubation in broth, retrieved the lipid via an extraction of the broth. As detailed in Table II, thin-layer chromatography of retrieved material incubated with broth previously used to support yeast growth revealed that the bulk of the radioactivity was distributed between two spots (72% at $R_f = 0.29$ and 26% at $R_f = 0.84$). These R_f values correspond to DMPC and myristic acid, respectively. When aliquots of the same sample of amphotericin

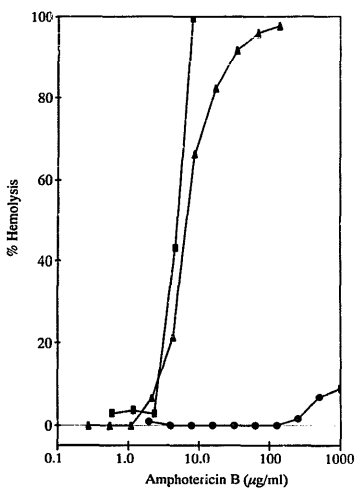


Fig. 6. Hemolytic activity of amphotericin B following 20 h incubation with a 4% RBC suspension at 37°C. The central fractions from each band of six 5 mole percent amphotericin B-lipid samples separated by density gradient were pooled to provide enough material for the measurements. The amphotericin B mole percentages of each of the two pooled populations were 1.2 mole percent and 37 mole percent for material taken from the low- (■) and high- (●) density bands, respectively. The profile of Fungizone ® (▲) is also shown. Note that the concentration scale is logarithmic.

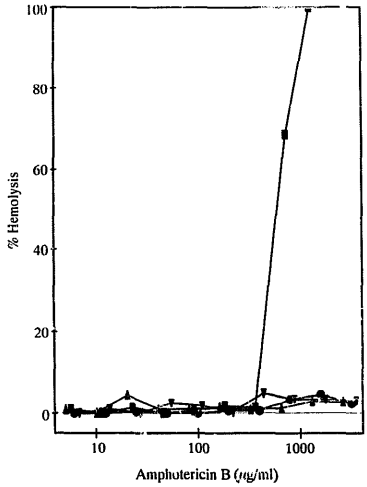


Fig. 7. Hemolytic activity of amphotericin B-DMPC/DMPG made up at 50 mole percent amphotericin B and exposed to either broth previously used to support yeast growth (■), exposed to broth never used to support yeast growth (●), exposed to broth used to support yeast growth that was heated to 95°C for 1 h prior to amphotericin B addition (▲), or not exposed to broth but kept in buffer solution (▼). For the cases where broth was used as a growth media for yeast, yeast cells were removed from the broth via low speed centrifugation before introduction of amphotericin B. The final mole percent amphotericin B for the abovementioned samples was 62, 52, 52 and 50, respectively. RBC hemolysis could be underestimated since monomeric amphotericin B that was released from the complex during incubation would not have been completely recovered.

B-DMPC/DMPG were incubated with either: (1) broth never used to support yeast, (2) used broth that had been heated (95°C for 60 min), or (3) buffer solution,

TABLE I

Minimal inhibitory concentrations (MICs) of amphotericin B mixtures with DMPC/DMPG and deoxycholate

For 5 mole percent amphotericin B in DMPC/DMPG (7:3), the high- and low-density fractions consisted of 33 and 1.5 mole percent amphotericin B, respectively. The deoxycholate preparation was Fungizone ®, a commercially available formulation.

	MIC (μg/ml)	
	C. albicans	*S. cerevisiae*
5 mole percent in DMPC/DMPG (separated)		
High-density material	0.16–0.32	1.25
Low-density material	0.16–0.32	1.25
25 mole percent in DMPC/DMPG in deoxycholate	0.16–0.32	0.63
	0.08–0.16	0.16–0.32

greater than 98% of the radioactivity migrated as DMPC. Thus, the apparent in vitro selective toxicity of the high-density material could be linked to a lipase-dependent remodeling of the drug-lipid complex. A similar mechanism involving remodeling of the amphotericin B-lipid complex with subsequent release of drug may also occur in vivo.

When DMPC/DMPG (7:3) liposomes containing no amphotericin B were incubated with broth, very little lipid breakdown could be detected. It is well documented that lipase activity varies significantly in saturated lipid systems depending upon the bilayer physical state [29–33]. At 37°C DMPC/DMPG liposomes are liquid crystalline while lipid in the amphotericin B-lipid complex is in a unique highly ordered putatively interdigitated phase [11,12]. This phase is

TABLE II

Percentage radioactivity found as fatty acid after incubation of [^{14}C]-DMPC / DMPG / amphotericin B in yeast-free broth

A is filtered broth that had been used to support yeast growth. B is broth never exposed to yeast. C is broth used to support yeast that was filtered and then heated to 95°C for 1 h. D is buffer solution. Numbers in brackets indicate the % of total radioactivity migrating on the TLC plate as DMPC. Filtration of the broth did not remove the extracellular species responsible for lipid breakdown since unfiltered broth used with the amphotericin B sample gave similar values (25% as myristic acid, 71% as DMPC).

	% total radioactivity found as myristic acid			
	A	B	C	D
DMPC/DMPG/ Amphotericin B (7:3:10)	26 (72)	0.7 (99)	2 (98)	1 (99)
DMPC/DMPG (7:3)	7 (92)	1 (98)	2 (98)	1 (99)

apparently more susceptible to lipase dependent degradation.

Lipid headgroup interactions

To further elucidate the nature of the interaction between lipid and amphotericin B we examined the contribution that the headgroup has upon formation of the ribbon-like structures. Comparing amphotericin B-single lipid systems, we found that formation of the high-density drug-lipid complex in low mole percent amphotericin B preparations was not restricted to the DMPC/DMPG (7:3) mixture but also occurred when amphotericin B was incorporated at 5 mole percent into either DMPC or DMPG. Two populations were resolved on sucrose density gradients for both lipid-amphotericin B systems (data not shown). Because of inherent differences in lipid density, the positions of the populations arising in the mixtures were shifted (DMPG ran lower on the gradient than DMPC mixtures). Even so, the distribution of amphotericin B between liposomal and high-density populations was similar to that of the typical combined lipid system where greater than half of the total amphotericin B was found in the high-density band. The equivalent ability of each lipid to interact with amphotericin B

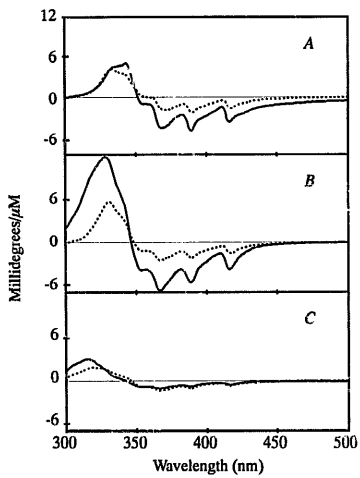


Fig. 8. CD spectra of amphotericin B prepared at (A) 5 mole percent, (B) 25 mole percent and (C) 50 mole percent in either DMPC (·····) or DMPG (———). Amphotericin B was 20 μM in all cases.

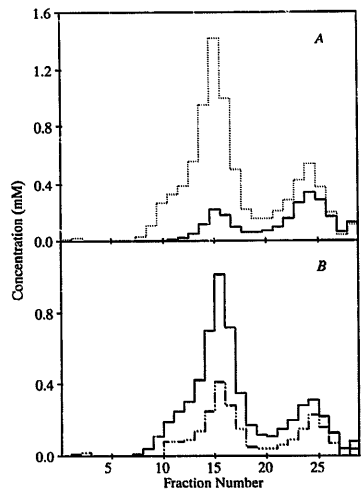


Fig. 9. Density gradient analysis of DMPC and DMPG distributions from a dispersion of DMPC/DMPG (7:3) with 22 mole percent amphotericin B. (A) Total lipid (·····) and amphotericin (B (———) distributions. (B) Distribution of DMPC (———) and DMPG (–·–·–) determined from [^{14}C]DMPC and total phospholipid distributions. Sample (0.2 ml of a 2 mg/ml amphotericin B) was placed atop a 5 ml 0–40% (w/v) sucrose gradient. 200-μl fractions were taken. A repeat experiment gave similar distributions (data not shown).

suggested that the headgroup interactions contribute little to the association that occurs in the high-density complex.

The CD spectra of amphotericin B at 5, 25, and 50 mole percent in either DMPC or DMPG are illustrated in Fig. 8. For both lipids, the broad positive peak centered near 320–330 nm was blue-shifted with increasing amphotericin B content. Signal amplitudes were consistently greater for DMPG-amphotericin B preparations. Except for the shape of the positive 320–340 nm absorption of 5 mole percent amphotericin B preparations, positions of the peaks were similar. These similarities reaffirm that formation of the complexed species is not headgroup dependent. To verify the possibility that preferential headgroup association could have occurred and remained undetected by spectroscopic techniques, we examined DMPC/DMPG ratios in various fractions derived from a 22 mole percent amphotericin B preparation at a 7:3 mole ratio of DMPC/DMPG (Fig. 9). Two bands were resolved which we have found to be typical of preparations made below 25 mole percent amphotericin B. The DMPC/DMPG ratio was only slightly higher than expected (72 mole percent DMPC) for fractions comprising the lower density population and only slightly lower than expected (68 mole percent DMPC) in the fractions containing the high-density population, where the ratio of amphotericin B/lipid approached 1:1. This again indicated that headgroup interactions play little role in the association between lipid and amphotericin B.

Discussion

The reduced toxicity exhibited by the high mole percent amphotericin B-lipid complex and its appearance within low mole percent preparations suggests that it alone is responsible for the benefits assigned 'liposomal' formulations. In fact, we found that truly liposomal material, liposomes bearing 1–2 mole percent amphotericin B (monomeric) that were separated from the complexed form by density gradient centrifugation, afforded no buffering of toxicity. Penetration of monomeric amphotericin B into the lipid hydrocarbon of a liposome would seem highly unfavorable due to the hydrophilic poly-hydroxyl region of the molecule. Amphotericin B monomers would therefore be restricted to the membrane interfacial region, where they would easily exchange with cellular material. Above local concentrations of approx. 3 mole percent, however, amphotericin B apparently is able to sufficiently disrupt individual lipid bilayers to be able to insert. Such insertion occurs concomitantly with the loss of liposomal structure, amphotericin B complexation and reduction in overall toxicity. Although details regarding the nature of this insertion are as yet unknown, the morphology of the resulting amphotericin B-lipid association can be described in terms of a membranous ribbon structure making a final collective organization other than bilayer difficult to imagine.

In fact, negative stain electron microscopy revealed that the ribbon-like structures are collapsed and aggregated membranes. Since only cross fractures were revealed by freeze-fracture electron microscopy we believe that the bilayer midplane, through which fracture faces are revealed, is absent and the amphotericin B-DMPC/DMPG membrane exists as an interdigitated bilayer. As has been suggested by others [37], such an arrangement would explain the high degree to which lipid is immobilized within amphotericin B-lipid mixtures [11,38]. Using wide angle X-ray diffraction, Lagner and co-workers [39,40] have shown that the 4.2 Å reflectance for fully hydrated DHPC is shifted to 4.1 Å on interdigitation of the acyl chains. We have observed a shift from 4.22 Å to 4.10 Å for DPPC bilayers that were induced to interdigitate by ethanol (Boni, L., Perkins, W., Minchey, S., Ahl, P., Slater, J., Gruner, S., Tate, M. and Janoff, A., submitted for publication). While not definitive for interdigitation, a similar shift (4.24 Å to 4.16 Å) occurred for DMPC/DMPG containing high mole percentages of amphotericin B [11].

One model for the complexation between amphotericin B and lipid depicts eight 1:1 drug-sterol units combining to form a circular pore [41]. In this arrangement the hydrophobic regions align with the lipid hydrocarbon chains and the amino sugars of the amphotericin B orient with the lipid headgroups. Although a similar bilayer arrangement of amphotericin B-phospholipid (1:1) may occur in the absence of sterol, no clear verifiable tests are available. However, DMPC and DMPG both possess fully saturated acyl chains which, in lieu of sterol, may act as a template for complexation. In the case of a fully interdigitated bilayer, the membrane thickness would approach half that of a normal bilayer. As a consequence, amphotericin B-lipid association would differ from that depicted in the model by De Kruijff and Demel [41]; an opposing orientation of the polar groups of lipid and amphotericin B would be required to minimize exposure of hydrophobic domains to the aqueous environment. Lipid headgroups and the amino end of amphotericin B might orient in the same or opposite direction or perhaps amphotericin B-lipid pairs within the complex alternate in direction (many configurations seem possible). Whatever the arrangement the membrane would possess two outwardly facing polar surfaces.

A 1:1 arrangement between lipid and drug is consistent with several observations. For example, Dufourc et al. [38] have noted a monotonic ordering of all positions along the acyl chain of DMPC in contact with amphotericin B. In fact, they found that immobilization

of deuterium-labeled DMPC by amphotericin B occurs at a 1:1 molar ratio. Similarly we have found that although we can achieve toxicity buffering at low concentrations of amphotericin B in lipid due to the spontaneous occurrence of high mole ratio complexes, a 1:1 stoichiometry between lipid and drug seems to be optimal in terms of toxicity reduction. Most likely the enhanced reduction in toxicity we find on increasing the concentration of amphotericin B from 25 to 50 mole percent in lipid results from a closer (more ordered) association of drug-lipid complexes within the bilayer structure. In fact, we showed previously that lipid could be 'squeezed' out of 25 mole percent amphotericin B-DMPC/DMPG by temperature cycling, with a concomitant reduction in toxicity, while 50 mole percent amphotericin B-DMPC/DMPG remained unaffected [11]. While the basic features of CD spectra were the same for both 25 mole percent and 50 mole percent amphotericin B-lipid complexes studied here, there was a slight blue shift of the broad positive peak located near 320 nm and a reduction in signal amplitude as amphotericin B content was increased. These differences likely reflect the changing interaction that occurs between complexes when border lipid between complexes is removed.

Madden et al. [36] demonstrated that amphotericin B partitioning from solution into DMPC/DMPG liposomes was dependent upon DMPG content. While DMPC is zwitterionic, DMPG is negatively charged at neutral pH. Although amphotericin B is also zwitterionic, its amino sugar is positioned at the end of the chain along which lipid binding is expected to occur. An electrostatic interaction between the lipid headgroup and the amino sugar likely occurred in these investigations, however, only liposomal material evolved (no high-density species was observed). Because we found no preferential binding of amphotericin B to either DMPC or DMPG in high-density material, we believe that electrostatic interactions are not important in the formation of the complexed amphotericin B-lipid species.

The reduced toxicity afforded by complexed amphotericin B apparently stems from restricted partitioning of amphotericin B into solution. We found that where partitioning should occur most readily, from monomers on liposomes and micellar material, amphotericin B toxicities were the greatest. Recently, Jullien et al. [34] found that the adverse effects of amphotericin B in vitro could be correlated with unbound amphotericin B that had partitioned out of the liposome. More cogently we find that the release of amphotericin B monomer from the complexed form, a phenomena crucial for its effectiveness as an antifungal agent, results from the remodeling (delipidation) of the complex in the presence of yeast lipases. In fact, phospholipase-deficient strains of yeast are somewhat resistant in vitro to complexed amphotericin B [35]. The metastability of the ribbon complexes in the biological milieu may explain both why efficacy is preserved and why toxicity can apparently be buffered to a greater extent in vitro than in vivo determinations would reflect [20].

In our experiments significant lipase-dependent degradation of DMPC/DMPG systems occurred only in the presence of amphotericin B. It is well known that in saturated lipid systems significant hydrolysis occurs only when lipid is near the gel-liquid crystalline phase transition or when liposomes are highly curved (small unilamellar vesicles) [29–33]. At the 37°C incubation temperature, our control DMPC/DMPG liposomes ($\geq$ 500 nm in diameter) were 14°C above their phase transition temperature (liquid-crystalline phase). Our previous results have shown that the physical state of amphotericin B-DMPC/DMPG is clearly different [11,12]. It is likely that the highly ordered, presumably interdigitated, amphotericin B-lipid structures possess packing defects similar to those occurring in lipid undergoing a phase transition and/or regions of high curvature. The latter is consistent with freeze-fracture EM results where sharp folds in the ribbon-like structures were apparent (see Fig. 5B).

In conclusion, the reduction in toxicity of amphotericin B in DMPC/DMPG was completely accounted for by the formation of a high mole percent drug phase composed of aggregates of a single drug-lipid complex. Liposomally associated amphotericin B on the other hand remained significantly toxic to RBCs and yielded a monomeric CD signature. Differences in amphotericin B toxicity to RBCs between the two types of association with lipid were dramatic and probably reflect a reduced exchange rate of drug from the macromolecular complex to the target cell. We believe that the stability of this macromolecular complex arises from an association of lipid and amphotericin B into a highly ordered putatively interdigitated membrane. In the presence of a cell-free broth previously used to support yeast growth the integrity of complexed material was compromised by what appear to be yeast lipases, thus rendering amphotericin B available for cellular destruction. This mechanism of release explains why similar in vitro activities toward yeast but not RBCs could be achieved for both liposomal and complexed amphotericin B. Inclusion of other lipophilic drugs into similar well-ordered packing arrangements with lipid may allow for a decrease in release rate, and toxicity, of those compounds.

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