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LIPOSOMAL AMPHOTERICIN B IS TOXIC TO FUNGAL CELLS BUT NOT TO MAMMALIAN CELLS

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Amphotericin B is an efficacious but extremely toxic anti fungal drug. Recently it has been shown that the incorporation of Amphotericin B in multilamellar liposomes results in a marked reduction in drug toxicity in mice with no loss of anti fungal potency. Until now, the mechanistic basis of the enhanced therapeutic index of liposomal Amphotericin B has been unclear. In this report, however, we show that the in vivo effects can be mimicked in vitro where free but not liposomal Amphotericin B causes lysis of erythrocytes while both free and liposomal drug kill fungal cells. These results suggest that the markedly improved therapeutic index of liposomal Amphotericin B is largely due to a fundamental alteration in the ability of the drug to interact with mammalian cell membranes rather than to alterations in pharmacokinetics or drug distribution.

Systemic fungal infections are a major source of morbidity and mortality in cancer patients and other immunocompromised individuals [1,2]. The current drug of choice for most systemic mycoses is Amphotericin B, a potent but extremely toxic agent. Amphotericin B is a lipophilic compound which interacts with ergosterol in fungal membranes thus creating transmembrane channels which permit the escape of vital ions and metabolites. Unfortunately, the drug also interacts appreciably with the cholesterol found in mammalian membranes and this is probably the basis of its profound toxicity to the kidney, hematopoietic system and central nervous system [3–5].

In recent years, a number of investigators have shown that the incorporation of certain drugs in phospholipid vesicles (liposomes) can markedly alter the pharmacokinetics, tissue distribution, metabolism and therapeutic efficacy of these compounds [6,7]. In particular, incorporation of Amphotericin B in phospholipid-ergosterol vesicles [8,9] or in pure phospholipid vesicles [10] results in a marked reduction in the toxicity of the drug to animals with apparent maintenance of therapeutic effect against parasitic [8] or fungal [9,10] infections.

In this report we explore the mechanistic basis of the enhanced therapeutic index of liposomal Amphotericin B. We demonstrate that the behavior of liposomal Amphotericin B in vivo can be mimicked in vitro. Thus, we show that whereas free Amphotericin B will cause lysis of mammalian erythrocytes, the liposomal drug fails to do this even at extremely high drug concentrations. By contrast, free and liposomal Amphotericin B are equally effective in killing fungal cells.

The method for preparing multilamellar liposomes containing Amphotericin B has been described previously [10]. Briefly, Amphotericin B (Squibb) dissolved in methanol was mixed with a chloroform solution of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) (Avanti Polar Lipids). Organic solvents were removed by evaporation under vacuum and the resultant lipid film was dispersed by hand shaking in 0.9% NaCl to form

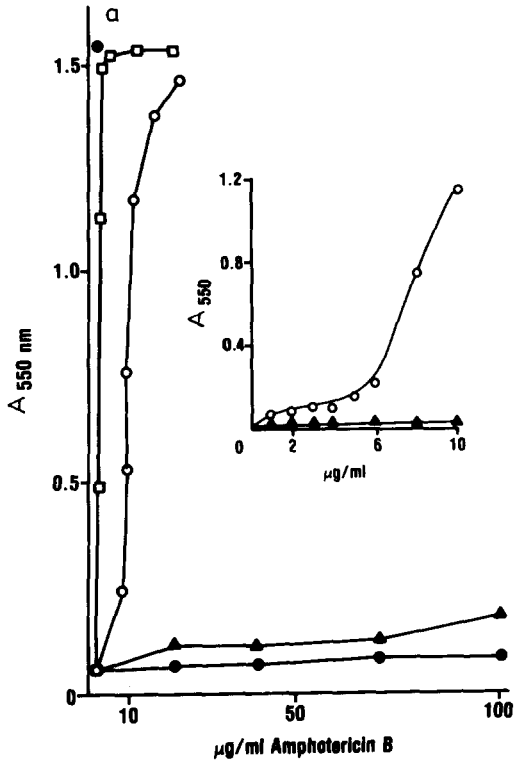
liposomes. The final preparation contained a 7 : 3 molar ratio of DMPC/DMPG and various amounts of Amphotericin B, which was quantitated by its absorbance at 405 nm after solubilization in chloroform/methanol. In some cases cholesterol or ergosterol were included in the liposomes. The stability of liposomal Amphotericin B was evaluated by incubating the vesicles in isotonic saline, yeast nitrogen base or conditioned yeast nitrogen base (supernatant of *Candida albicans* culture at 10^8 colony forming units (c.f.u.)/ml). The vesicles were then sedimented (10000 rpm, 20 min) and the residual Amphotericin B measured by absorbance at 405 nm after solubilization in chloroform/methanol.

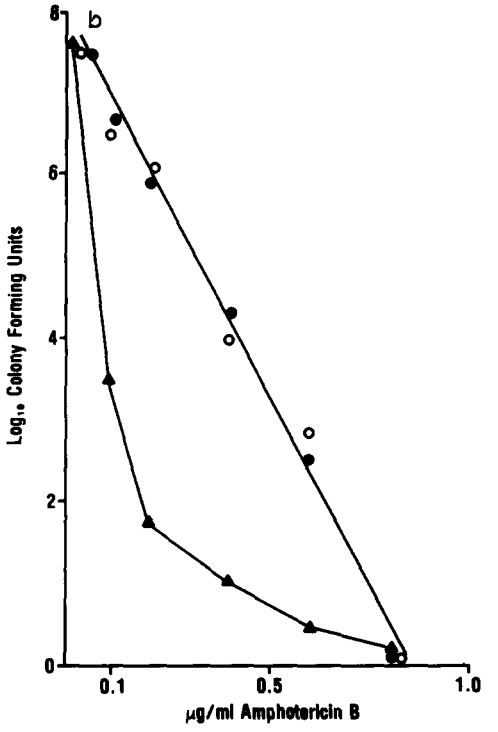
For studies of effects of free or liposomal Amphotericin B on mammalian cells, fresh, washed human red cells were added, at a final concentration of 2%, to tubes containing free Amphotericin B, liposomal Amphotericin B, or free drug plus liposomes, each suspended in saline [11]. The free Amphotericin B was originally made up in dimethyl formamide which was then present in all assays at a level of 4%. The samples were incubated for predetermined time intervals at 37°C, centrifuged at $10000 \times g$ for 20 min to remove vesicles and cells or cell debris and the released hemoglobin in the supernatant was determined by its absorbance at 550 nm; release of hemoglobin by hypotonic lysis of the same number of cells in H_2O was also measured. It should be noted that the amount of remaining free Amphotericin B, after addition of *Candida* or erythrocytes, was not determined in these experiments.

Studies of effects of free or liposomal AMB on yeast cells were conducted as follows. Stock cultures of *Candida albicans* strain 336 were diluted to $7 \cdot 10^5$ c.f.u./ml in yeast nitrogen base. Samples were mixed with various doses of free Amphotericin B, liposomal Amphotericin B, or free drug plus 'empty' liposomes and were incubated at 37°C for 16 h. Samples were then diluted in yeast nitrogen base, plated and subsequently the number of colonies for each dose was determined [12].

As we have described previously [10], intravenously administered liposomal Amphotericin B is far less toxic to mice than is the free drug. However, liposomal Amphotericin B remains an effective anti fungal agent. Treatment of *Candida*-infected mice with liposomal Amphotericin B had essentially the same effect as an equivalent amount of free drug, while treatment with 'empty' liposomes had no effect on survival [10]. Thus free and liposomal Amphotericin B are approximately equipotent in treating disseminated candidiasis in mice. However, since the liposomal drug is far less toxic, it is quite possible to administer drug doses which far exceed the LD_{50} for free Amphotericin B and which result in a marked improvement in the survival of infected mice [10]. At this point the mechanisms underlying the reduced toxicity and enhanced therapeutic index of liposomal Amphotericin B remained unclear. It is well known that the incorporation of a drug into liposomes can affect its behavior in vivo in a variety of complex ways including alteration of pharmacokinetics, changes in tissue distribution and modification of immune function [6,7]. However, we were surprised to find that the behavior we observed in vivo, namely an enhanced therapeutic index or improved selective toxicity of liposomal Amphotericin B, could apparently be mimicked quite precisely in vitro. That is, a comparison of the effects of free and liposomal Amphotericin B in vitro also demonstrated an enhanced selectivity of the liposomal drug in the form of reduced toxicity to mammalian cells with full retention of antifungal potency.

It is well known [4,11] that doses of Amphotericin B in the μg/ml range can readily lyse human erythrocytes as well as other types of mammalian cells. However, we found that when the Amphotericin B is present in phospholipid vesicles it has essentially no lytic effect on human erythrocytes even at extremely high doses (Fig. 1a). If liposomes are added to the free drug prior to the addition of erythrocytes, only a small amount of hemolysis occurs; presumably the liposomes can sequester the drug and prevent its interaction with the erythrocytes. By contrast, incorporation of Amphotericin B in liposomes has little effect on its ability to kill *C. albicans* in vitro; thus both free and liposomal drugs are approximately equipotent (Fig. 1b). Indeed, the addition of liposomes to free Amphotericin B prior to the addition of *Candida* cells actually seems to enhance the antifungal potency of the drug; the mechanism of this effect is unknown. Thus the liposomes seem to





sequester the drug in a manner which makes it unavailable and non toxic to mammalian cells but readily available and toxic to fungal cells.

In Fig. 2 we compare the effects of three different types of vesicle preparations on erythrocyte lysis in the presence and absence of Amphotericin B. Vesicles composed solely of phospholipids and those composed of phospholipids plus cholesterol protected red cells against Amphotericin B-induced lysis even during prolonged incubation (24 h). However, ergosterol-containing vesicles caused considerable cell lysis even in the absence of Amphotericin. This is in contrast to a number of observations indicating that ergosterol can protect fungal cells from Amphotericin-mediated cytoxicity [13,14]. It is well known that red cell sterols can exchange with exogenous sterols [15]; perhaps the exchange of ergosterol into the red cell membrane leads to a destabilization of the cell. The toxicity of ergosterol containing vesicles to mammalian cells suggests that vesicles composed solely of phospholipids [10] or phospholipids plus cholesterol may be preferable to ergosterol vesicles [8,9] for therapeutic purposes.

Fig. 1. (a) Lysis of human erythrocytes by free versus liposomal Amphotericin B. Washed human red cells were incubated with free Amphotericin B, with Amphotericin B incorporated in DMPC/DMPG multilamellar liposomes or with Amphotericin premixed with 'empty' DMPC/DMPG liposomes. The incubation was performed at 37°C, in phosphate-buffered saline, for predetermined time intervals. Lysis of red cells was quantitated by release of hemoglobin ($A_{550\ nm}$) as described in Methods. ○———○, free Amphotericin B 45 min incubation; □———□, free Amphotericin B 18 h incubation; ●———●, liposomal Amphotericin B (total lipid = 10 mg/ml) at 45 min; ▲———▲, free Amphotericin B premixed with 'empty' liposomes (total lipid = 10 mg/ml) at 45 min. Note: After 18 h incubation with liposomal Amphotericin B the degree of erythrocyte lysis did not exceed that in the buffer control. (Inset) Lower dose range. ○———○, free Amphotericin B 45 min incubation; ▲———▲, free Amphotericin B premixed with 'empty' liposomes 45 min incubation. (b) Killing of *Candida albicans* by free or liposomal Amphotericin B. *C. albicans* ($7 \cdot 10^5$) c.f.u./ml in yeast nitrogen base were incubated with free Amphotericin B, liposomal Amphotericin B or free Amphotericin B premixed with 'empty' liposomes for 16 h at 37°C. The cultures were then diluted in yeast nitrogen base and the number of surviving colonies on agar for each dose was determined. ○———○, free Amphotericin B; ●———●, liposomal Amphotericin B (total lipid = 10 mg); ▲———▲, free Amphotericin B, premixed with 'empty' liposomes (total lipid = 10 mg).

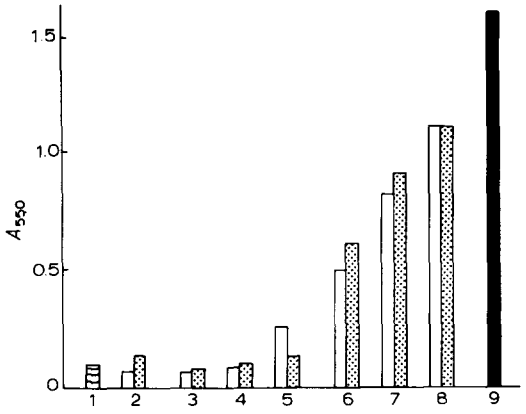


Fig. 2. Effect of vesicle composition on the lysis of erythrocytes. Fresh human red cells were incubated with buffer, with free Amphotericin B (42 μg), with Amphotericin B incorporated into lipid vesicles (38–45 μg Amphotericin B, 10 mg total phospholipid) or with 'empty' vesicles (10 mg total phospholipid) for 24 h at 37°C and the degree of erythrocyte lysis was determined as in Fig. 1a. Vesicles were composed of DMPC and DMPG in a 7:3 molar ratio with the addition of various amounts of cholesterol (C) or ergosterol (E). Results are the means of triplicate determinations. 1, phosphate-buffered saline; 2, pure phospholipid (PL); 3, PL/C 10:2; 4, PL/C 10:4; 5, PL/C 10:8; 6, PL/E 10:2; 7, PL/E 10:4; 8, PL/E 10:8; 9, free Amphotericin B. Open columns, 'empty' vesicles, shaded columns, vesicles containing Amphotericin B.

Although other investigators have demonstrated protective effects of lipids, including sterols, against the action of polyene antibiotics, to our knowledge no one has previously demonstrated that these effects can be selective in the sense of protecting mammalian cells to a greater degree than fungal cells. Our observations are not explainable by artifactual release of free drug from the liposomes upon incubation with *C. albicans*. Prolonged incubation of Amphotericin B liposomes with yeast medium or with fungal cells does not result in destabilization of the liposomes nor in release of the drug. Chemical determination (Table I) indicates that over 90% of the Amphotericin B remains with the vesicles composed of phospholipid or phospholipid plus sterols under these circumstances; moreover, supernatants of vesicles incubated overnight with conditioned yeast medium are not toxic to *Candida*.

Our results indicate that Amphotericin B can be transfered efficiently from liposomes to fungal cells, but not to mammalian cells. The precise mechanism of this transfer remains undefined at present. A direct or 'collisional' transfer seems possible since drug effects on *Candida* take place in the absence of detectable drug leakage from the liposomes. However, differences in the rate of diffusional transfer from 'donor' liposomes to the highly dissimilar membranes of red cells versus fungal cells might also account for our observations [16]. Nonetheless, it seems clear that the incorporation of the polyene antibotic Amphotericin B in phospholipid vesicles results in a profound alteration of the pattern of interaction of the drug with fungal and mammalian cell membranes, and that this may account, in part, for the enhanced therapeutic index of liposomal Amphotericin B observed in vivo.

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TABLE I

STABILITY OF AMPHOTERICIN B-CONTAINING LIPOSOMES

Incubation temperature was 37°C. The amount of Amphotericin B was determined spectrophotometrically at 405 nm. Results are the means of duplicate determinations, differing by less than 1%.

Liposome composition	Percent release of Amphotericin B in			
	Yeast nitrogen base		Conditioned yeast nitrogen base [a]	
	24 h	48 h	18 h	36 h
Phospholipid (DMPC/DMPG, 7:3)	0	0	4	4
Phospholipid + cholesterol (7:3:2)	0	3.6	–	–
Phospholipid + ergosterol (7:3:2)	3.9	7.0	–	–

[a] Yeast Nitrogen Base was conditioned by overnight incubation with *Candida albicans* or liposomes or both before adding Amphotericin B containing liposomes.

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