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Structure-Activity Study of Inhibition of Amphotericin B (Fungizone) Binding to Sterols, Toxicity to Cells, and Lethality to Mice by Esters of Sucrose

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The effects of four monoesters of sucrose with different acyl chain lengths (palmitate, C_{16}; myristate, C_{14}; laurate, C_{12}; and caprate, C_{10}) on the aggregation state of amphotericin B (AmB), its binding to cholesterol and ergosterol, its toxicity to cells, and its lethality to mice were determined. In solution, all four of these esters inhibited AmB binding to cholesterol more than to ergosterol; this effect correlated with the ester-induced shift from the mainly aggregated form of AmB to the mainly monomeric form. In experiments with cells, the esters inhibited the toxicity of AmB to mouse erythrocytes and cultured mouse fibroblast L-929 cells more than its toxicity to *Candida albicans* cells. When injected intravenously with AmB, these esters decreased AmB lethality to mice. In all of these assays, the ester with the shortest chain length (caprate) was much less potent than the other three esters. Our results indicate a correlation between in vitro and in vivo assays and suggest that the in vitro and in vivo selectivity of AmB may be enhanced by surface-active agents which modulate the aggregation state of AmB.

Amphotericin B (AmB) combined with deoxycholate (Fungizone) is the principal antifungal agent for most systemic fungal infections. However, the significant toxicity associated with its use imposes limits on its clinical efficacy. As a result, various strategies have been developed to try to circumvent this problem. A major focus of these efforts has been to examine novel delivery systems which might result in a decrease in AmB toxicity to the host without significantly impairing its antifungal activities (12, 14).

It has been proposed that the lower toxicity to mice of AmB complexed to lipids, as compared with the toxicity of free AmB, is linked to the lower toxicity of AmB-lipid preparations to host cells (9, 13). The toxicity of AmB to cells is, in turn, thought to occur as a result of its binding to membrane sterols (2). Recently we have reported that a relatively mild detergent, sucrose laurate, strongly inhibited AmB binding to cholesterol and AmB toxicity to mammalian cells (7). In the present work, we investigated the effect of acyl chain length of sucrose esters on their capacity to inhibit AmB binding to sterols and in vitro and in vivo toxicities. We compare here the results obtained for four sucrose esters: caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), and palmitate (C_{16}). All esters inhibited AmB lethality to mice, and the extent of this effect was linked to their in vitro action.

MATERIALS AND METHODS

Chemicals. Cholesterol and ergosterol were purchased from Sigma, St. Louis, Mo., and were crystallized twice from ethanol. AmB as Fungizone was purchased from E. R. Squibb & Sons, Princeton, N.J. Fungizone is available as a sterile lyophilized powder in vials providing 50 mg of AmB and 41 mg of sodium deoxycholate with 25.2 mg of sodium phosphate as a buffer. Sucrose laurate (catalog number L-1685) and sucrose caprate (catalog number SM-1000) were generous gifts of Mitsubishi-Kasei Food Corp., Tokyo, Japan. Sucrose palmitate and myristate were synthesized by us according to the method of Osipov et al. (15). Each compound showed a single spot on thin-layer chromatograms (silica gel), and its structure was confirmed by infrared and nuclear magnetic resonance spectra.

Spectroscopic experiments. Solutions of AmB, sterols, and sucrose esters were prepared as described previously (7). Absorption spectra were recorded on a Varian 634 double-beam spectrophotometer.

Cellular experiments. Erythrocytes were separated from mouse blood obtained by orbital bleeding. Erythrocytes and *Candida albicans* cells (strain B_{311}) were used to assay the effects of the sucrose esters alone and in combination with AmB as described previously (7). The cell-associated K^+ was measured in a flame photometer, and the hemoglobin remaining in cells was measured spectroscopically; the viability of fungal cells was measured by plating cells on Sabouraud agar and counting colonies after 48 h of incubation.

Mouse fibroblast L-929 cells were cultured in monolayers in plastic dishes; they were assayed for the cell-associated K^+ as described previously (7).

Animal experiments. A/J female mice (6 weeks old) purchased from Jackson Laboratory were housed for 1 week prior to the onset of the experiments. Solutions of AmB, sucrose esters, or AmB-sucrose ester combinations were prepared in 5% glucose. To measure lethality, groups of 10 mice were given, over 2 min, intravenous injections of 0.2 ml of the preparation to be assayed. The doses were adjusted according to the average weight of the animals, measured just before the start of the experiments. Acute toxicity was assessed by determining the number of deaths over 24 h following the injection. The dose which resulted in death for

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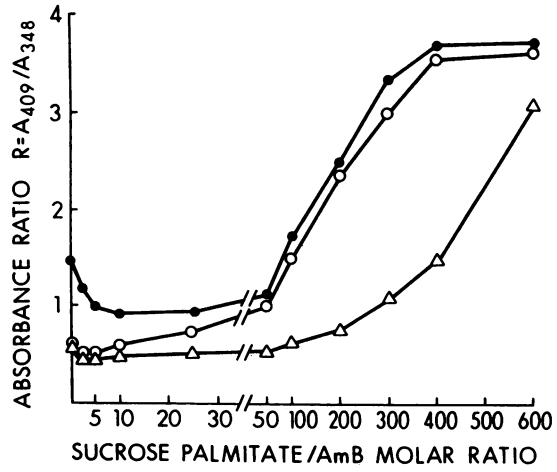

FIG. 1. Effects of sucrose palmitate on ratios of absorbances of 6.5×10^{-6} M AmB dispersed in 6.5% propanol in the absence of sterols (●) or in the presence of 6.5×10^{-6} M cholesterol (○) or 6.5×10^{-6} M ergosterol (△).

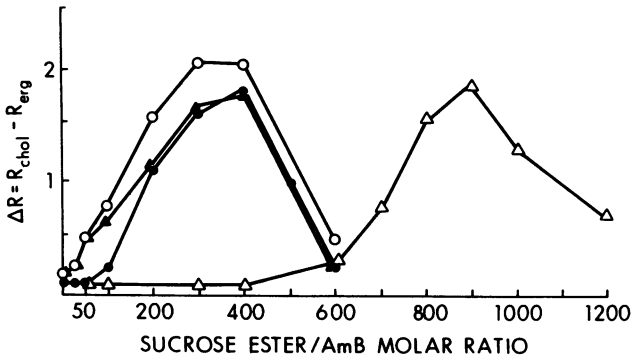

FIG. 2. Effects of sucrose palmitate (○), myristate (▲), laurate (●), and caprate (△) on the selectivity of AmB binding to cholesterol and ergosterol. AmB and sterol concentrations were as described in the legend to Fig. 1.

50% of the mice (LD_{50}) was estimated from the dose-response curve.

RESULTS

Effect of sucrose esters on AmB binding to cholesterol and ergosterol. AmB binding to sterols and its self-aggregation are reflected by changes in its UV-visible absorption spectrum. The ratio of A_{409} to A_{348} may be used to monitor these two phenomena (3,7). We have recently reported (3, 7) that sucrose laurate used in mixtures with AmB at concentrations higher than 0.01% induced a shift from the mainly aggregated form of AmB to the monomeric form. This effect was also dependent on the AmB concentration. At the same range of concentrations of sucrose laurate, an inhibition in the binding of AmB to cholesterol was observed. Binding to ergosterol was much less inhibited. For the present study, we have used the same spectral analysis to determine the effects of three other sucrose esters, caprate, myristate, and palmitate, on the binding of AmB to cholesterol and ergosterol. Figure 1 shows the absorbance ratio R (A_{409}/A_{348}) of the AmB spectrum as a function of the concentration of sucrose palmitate for AmB alone and in the presence of equimolar concentrations of ergosterol or cholesterol. When AmB alone was used, the ratio R decreased at low palmitate concentrations, leveled off, and began to increase at palmitate/AmB molar ratios higher than 50; at a palmitate/AmB molar ratio of 400, R reached the value of about 4, which is characteristic of monomeric AmB. Addition of ergosterol in concentrations equimolar to those of AmB induced a decrease in R over the whole range of the ester concentrations, whereas addition of equimolar concentrations of cholesterol did not significantly change the spectrum of AmB in the presence of similar concentrations of the sucrose ester. Thus, the increase in the proportion of AmB in the monomeric state was paralleled by a decrease in AmB-cholesterol interaction.

The same kind of behavior was observed for sucrose myristate and caprate. Thus, all sucrose esters studied modulate AmB binding to sterols in the same way. The inhibition of this binding to cholesterol was much stronger than to ergosterol and was seen at the same concentrations of esters which induced for AmB alone a shift to the mainly monomeric form. A comparison of the effects induced by the four esters is represented in Fig. 2. Here a plot of ΔR (R with cholesterol minus R with ergosterol) as a function of the sucrose ester/AmB molar ratio is shown. ΔR may be considered as a measure of the selectivity in the AmB binding to the two sterols. The maximal value of ΔR is similar for all esters (Fig. 2). For a constant AmB concentration, the concentrations of esters required for induction of this maximal selectivity are similar for palmitate, myristate, and laurate and about twice as high for caprate. Similar results were obtained when AmB without deoxycholate was assayed. In these experiments, the concentrated solution of AmB in dimethyl sulfoxide was added to the medium.

Inhibition of AmB toxicity to cells by sucrose esters. The toxicities of AmB, sucrose esters, and AmB-ester combinations to mammalian cells were measured as a decrease in the retention of K^+ by erythrocytes and L cells and as a loss of hemoglobin by erythrocytes. The concentrations of esters effective in inducing decreases in K^+ and hemoglobin contents of erythrocytes were comparable. Figure 3 illustrates ester induced decreases in retention of K^+ by erythrocytes and L cells. In both groups of experiments, the toxicities of sucrose palmitate and myristate were similar, about 10 times greater than that of sucrose laurate and about 100 times greater than that of sucrose caprate.

When the toxicities of AmB-ester combinations to cells were measured, concentrations of AmB which produced a decrease in measured indices to 5 to 25% of control values were used. Figure 4 shows the effects of sucrose esters on AmB-induced decreases in retention of K^+ and hemoglobin by erythrocytes and retention of K^+ by L cells. In all three assays, the order of inhibitory potency was the same as that observed for the order of toxicity: palmitate ≥ myristate > laurate > caprate. Maximal ester-induced protection from the AmB toxicity was comparable for palmitate and myristate, about equal or slightly lower for laurate, and the lowest for caprate. Further increases in concentrations of esters resulted in progressive increases in toxicity.

Although the general dose-dependent patterns of the toxic and inhibitory actions of all four esters were similar, the protective effects of laurate were seen over a larger range of ester concentrations than were those of palmitate and myristate. This characteristic of sucrose laurate may be of importance if sucrose esters are considered candidates for in vivo studies.

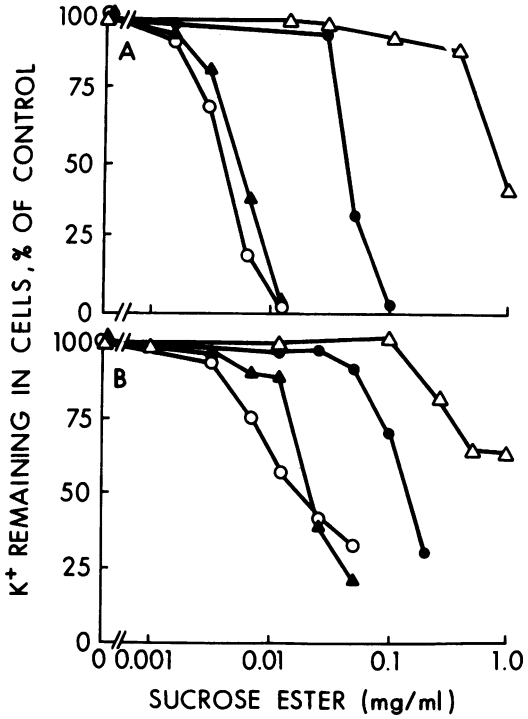

FIG. 3. Effects of sucrose palmitate (○), myristate (▲), laurate (●), and caprate (△) on retention of K^+ by erythrocytes (A) and L cells (B). Data are from one representative experiment. Similar results were obtained in two other experiments.

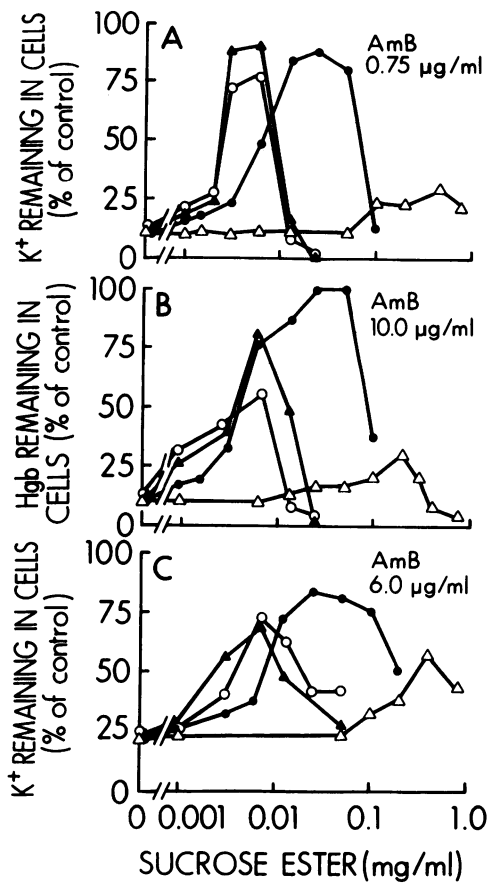

FIG. 4. Effects of sucrose palmitate (○), myristate (▲), laurate (●), and caprate (△) in combination with AmB on retention of K^+ (A) and hemoglobin (B) by erythrocytes or retention of K^+ by L cells (C). Data are from one representative experiment. Similar results were obtained in two other experiments.

The toxicities of AmB, sucrose esters, and AmB-ester combinations to *C. albicans* cells were measured as a decrease in the retention of K^+. At concentrations up to 2.5 mg/ml, the sucrose esters were not toxic to fungal cells (data not shown). Figure 5 shows that the inhibitory action of esters of sucrose on the AmB-induced decrease in retention of K^+ by *C. albicans* cells increases progressively with increases in the ester concentrations. The order of the inhibitory potency of the esters was the same as that observed for inhibition of AmB toxicity to mammalian cells (palmitate > myristate > laurate > caprate), but the concentrations required to induce the inhibition were much higher in the case of *C. albicans*.

From the data presented in Fig. 4 and 5, we can evaluate the most effective molar concentration of each ester, i.e., the concentration which increases the K^+ retention by AmB-treated cells from 5 to 25% to 55 to 75% of control values. The ratio of this most effective concentration of sucrose ester to the molar concentration of AmB used in given experiment may be considered an inhibitory index. These indices were several hundred times greater for fungal cells than for mammalian cells.

Inhibition of AmB lethality to mice by sucrose esters. Lethality was determined as LD_{50}s. The LD_{50}s of esters used as single agents determined in two similar experiments were 130 (myristate), 210 (laurate) and 320 (caprate) mg/kg. Thus, the pattern of toxicity of esters to mice was similar to the pattern of toxicity to mammalian cells: both decreased with the decrease in the length of the ester acyl chain. These results are in agreement with data in the literature: toxicity of nonionic surfactants increases with increases in the size of the lipophile (5).

The LD_{50}s obtained for AmB alone or in combination with esters are presented in Table 1. The combinations assayed were composed of several concentrations of AmB and three concentrations of esters (approximately one-fourth, one-half, and close to the whole LD_{50} of the ester). The LD_{50} of AmB used in combination with esters at doses corresponding to one-fourth of their LD_{50}s or close to their LD_{50}s did not increase or increased no more than 30% over the value found for AmB used alone. In contrast, the LD_{50} of AmB in combination with any ester, used at doses equivalent to half of its LD_{50}, was about twofold greater than the LD_{50} of AmB used alone.

TABLE 1. Acute toxicity to mice of AmB alone or in combination with sucrose esters

Ester	LD_{50} (mg/kg ± SE)[a] of AmB with an ester concn (mg/kg) of:				
	0	50	100	150	200
None	2.4 ± 0.4				
Myristate		5.8 ± 0.6	3.1	ND	
Laurate		3.5	5.2 ± 0.5	2.7	
Caprate			2.7	5.0 ± 0.5	1.3

[a] Values without standard errors are data from one experiment; similar results were obtained in another experiment. Values with standard errors are means from three separate experiments. ND, Not done.

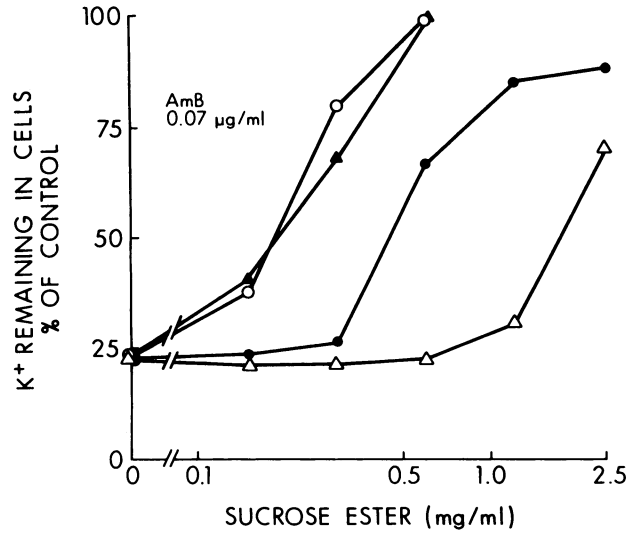

FIG. 5. Effects of sucrose palmitate (○), myristate (▲), laurate (●), and caprate (△) in combination with AmB on retention of K^+ by *C. albicans* cells. Data are from one representative experiment. Similar results were obtained in two other experiments.

DISCUSSION

Because toxicity of AmB is the main limitation of its use in treatment of life-threatening fungal infections, the search for a new, less toxic formulation is very important. It has recently been reported that phospholipid vesicles (14), oil emulsion (4), or cholesterol dispersions (16) lower AmB toxicity without affecting its antifungal properties. Despite an effort to understand the molecular basis of this selectivity (8–10), the issue is not yet settled.

Moreover, although the general association between the inhibitory effects of lipids on AmB toxicity to mammalian cells and its toxicity to animals was reported previously (9, 13, 17), a poor correlation between the inhibitory potencies of lipid vesicles in in vitro and in vivo systems has been found (17). In an attempt to gain an understanding of the molecular basis of the effects of the vehicle on in vitro and in vivo activities of AmB, we examined the effects of esters of sucrose on the physical state of AmB and its binding to sterols, toxicity to cells, and lethality to mice.

We used the four esters with acyl chains of differents lengths: (palmitate, C_{16}; myristate, C_{14}; laurate, C_{12}; and caprate, C_{10}) and we found that all of these esters induced a shift of AmB from its mainly aggregated to its mainly monomeric form. The concentrations of the esters required for comparable changes in the AmB aggregation state were similar for sucrose laurate, myristate, and palmitate and more than twofold greater for caprate.

It has been proposed that AmB binds more avidly to ergosterol than to cholesterol because of differences in the structure of the two sterols (1). In our experiments, however, no difference was seen in AmB interaction with ergosterol or cholesterol when it took place in the absence of sucrose ester; in the presence of sucrose esters, the binding of AmB to cholesterol was inhibited under the same conditions which allowed its binding to ergosterol (Fig. 1).

This selectivity was first observed at ester concentrations which induced a shift to the monomeric form of AmB and was maximal when AmB was almost entirely monomeric. Further increases in ester concentrations also resulted in the inhibition of AmB binding to ergosterol. A similar pattern of selectivity in the AmB-sterol interactions was found when sodium deoxycholate was used. However, the concentrations of deoxycholate which induced the AmB-sterol selectivity were much higher than the concentrations used in the commercial form of AmB, Fungizone (18).

The notion that the state of aggregation of AmB is a determining factor in its interaction with ergosterol has been previously reported by some members of our group in a study of propanol-water mixtures (6). Here this notion is confirmed and extended to cellular and in vivo studies.

The existence of a link between the aggregation state of AmB and its biological activities has already been postulated by others (9, 11). Our results give experimental support for these previous hypotheses.

AmB toxicity to cells is thought to originate from AmB binding to membrane sterols. The current observation that sucrose caprate, the least potent of the four esters in inhibiting AmB binding to cholesterol, was also the least potent in inhibiting AmB toxicity to cells supports this notion.

The concept that the greater decrease in AmB toxicity to mammalian cells compared with that in AmB toxicity to fungal cells induced by sucrose laurate is linked to the greater decrease in AmB binding to cholesterol compared with that in AmB binding to ergosterol has already been formulated by us (7). The present results extend this relationship to three other esters and link the ester-induced increase in the proportion of AmB in the monomeric state to the selectivity of AmB toxicity to mammalian and fungal cells.

The inhibitory potencies of esters of sucrose on AmB lethality to mice increased with the increase in the length of the acyl chain: myristate > laurate > caprate, the same order of activity which is seen in the inhibition of toxicity of AmB to cells. This suggests that the inhibition of AmB toxicity to mice is related to the inhibition of AmB toxicity to cells. By following this line of thinking, we can now propose that ester-induced decreases in AmB lethality to mice are associated with the aggregation state of AmB.

Obviously, the potential clinical use of sucrose esters as vehicles for AmB in the treatment of mycoses is limited by the toxicity of esters. Unfortunately, sucrose esters are more toxic to animal cells than to fungal cells and are toxic when injected into mice. However, our results in this study justify further testing of other surfactants on the aggregation state of AmB and its in vitro and in vivo selectivity.

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