ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1992, p. 2518–2522
0066-4804/92/112518-05$02.00/0

Vol. 36, No. 11

Effects of Aggregation and Solvent on the Toxicity of Amphotericin B to Human Erythrocytes

PHILIPPE LEGRAND,[1] ENEIDA A. ROMERO,[2] B. ELEAZAR COHEN,[2] AND JACQUES BOLARD[1]*

Laboratoire de Physique et Chimie Biomoléculaire (Unité Associée Centre National de la Recherche Scientifique 198), Université Pierre et Marie Curie, Tour 22, 4 Place Jussieu, 75252 Paris Cedex, France,[1] and Centro de Biologia Celular, Facultad de Ciencias, Universidad Central de Venezuela, Caracas 1041, Venezuela[2]

Received 2 December 1991/Accepted 13 August 1992

In aqueous suspensions of amphotericin B (AmB), a polyene antibiotic and antifungal agent, three forms of AmB coexist: monomers, water-soluble oligomers, and non-water-soluble aggregates. The toxicity of the water-soluble self-associated form of AmB compared with that of the non-water-soluble self-associated form was tested by measuring induction of K^+ leakage from human erythrocytes, using different suspensions containing the antibiotic and phosphate-buffered saline. These suspensions were obtained from various stock solutions of the antibiotic in dimethyl formamide or dimethyl sulfoxide. Their circular dichroism spectra around 340 nm, indicative of the degree of AmB self-association, were strongly dependent on the concentration of organic solvent in the suspensions. The nonsoluble self-associated form was separated from the water-soluble form by centrifugation. The nonsoluble form was favored by a high concentration of AmB of the stock solution. The kinetics of AmB-induced K^+ leakage from human erythrocytes also appeared to be strongly dependent on the AmB concentration of the stock solution being much weaker with concentrated stock solutions. It was concluded that the only form of AmB toxic to human erythrocytes is the water-soluble self-associated form (in contrast with fungal cells on which the monomeric form is also active). This result may be important in the design of new less toxic AmB derivatives and in the understanding of the mechanism of action of liposomal AmB.

Amphotericin B (AmB) remains the drug of choice for treating most systemic fungal infections. The recent introduction of liposomal AmB formulations with lower toxicity to patients (see, for instance, reference 5), has increased the clinical interest in this drug. However, its mechanism of action is still imperfectly understood. Inducing permeability to K^+ is thought to be the first event following the addition of AmB to cells and is therefore an indicator of its activity. In order to understand the mechanism of its selectivity between fungal (ergosterol-containing membranes) and mammalian (cholesterol-containing membranes) cells, several studies have been performed from the functional point of view with mammalian erythrocytes (see references 2 [and references therein], 6, 26, and 32) as well as from the molecular point of view (3, 29, 30, 33). In a previous study (3), we were able to show that AmB induces permeability to K^+ in cholesterol-containing membranes, particularly in erythrocytes only when AmB is in the self-associated form in the incubation medium. The threshold of concentration beyond which AmB self-association starts therefore appeared to be a determining factor in the activity of the drug. This point is very important for the design of new less toxic derivatives of AmB. It can also be a clue for the mechanism by which the selectivity of the drug is increased when it is incorporated into liposomes.

However, a more detailed analysis of the toxicity of the self-associated form was necessary, because it is well-known (23) that two self-associated forms are found in water, one water-soluble and one non-water-soluble. In the present study, we were able to modulate the ratio of these two forms and to correlate it with the induction of permeability to K^+ in human erythrocytes. Indeed, studies on the mechanism of action of AmB are generally performed with stock solutions of AmB in organic solvents (dimethyl formamide [DMF] or dimethyl sulfoxide [DMSO]), from which the drug is dispersed in the aqueous buffer used for biological studies. Self-association (leading to oligomeric species) and aggregation (of the oligomeric species) of the drug then occurs, which may be affected by the concentration of AmB in the stock solution. This possibility is investigated in the present study, by taking the amplitude of the AmB signal of circular dichroism (CD) around 340 nm as an indicator of self-association and correlating it with the induction of permeability to K^+ for different stock solutions. We showed that the concentration of AmB in the organic stock solution strongly affects the following: (i) the proportions of the different forms of AmB present in water as well as their nature and (ii) the toxicity of the drug to cholesterol-containing membranes, as monitored by the K^+ induction of permeability of human erythrocyte membranes.

Toxicity seems to be restricted to water-soluble self-associated AmB (oligomers), with monomeric AmB and non-water-soluble self-associated AmB (aggregates of oligomers) being inactive.

MATERIALS AND METHODS

Materials. AmB was a generous gift from Squibb France, Neuilly, France. Stock solutions of AmB were prepared with DMSO (Fluka, Buchs, Switzerland) or DMF (Touzart et Matignon, Vitry-sur-Seine, France) and used on the same day. Concentrations of AmB were determined by UV absorption after a 100-fold dilution in methanol (Prolabo, Paris, France) (ε at 407 nm = 150,000 M^{-1} cm^{-1}). Solvents were of first grade quality.

* Corresponding author.

Isolation of human erythrocytes. Human venous blood collected in tubes containing EDTA was centrifuged at 1,500 × *g* for 10 min, and plasma and buffy coat were removed. Erythrocytes were then washed three times with phosphate-buffered saline (PBS) (155 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4; adjusted to pH 7.4), suspended in PBS at an hematocrit of 40%, and used on the same day.

Measurement of antibiotic-induced K^+ leakage from erythrocytes. Appropriate amounts of AmB dissolved in organic solvent were added to PBS at 37°C. The suspensions were immediately mixed by vortexing and incubated for 5 min at the same temperature. Erythrocytes were then added to a final hematocrit of 2% (approximately 2×10^8 cells per ml) and incubated at the same temperature. For kinetic measurements, samples were taken at 5, 10, 15, 25, and 60 min. For dose-response curves, the incubation time was 1 h. Each sample was rapidly squirted into an ice-cold Eppendorf tube containing 0.1 ml of di-*n*-butyl-phthalate. The suspension was immediately centrifuged at 4,000 × *g* for 3 min, and the cell pellet which had passed through the oil cushion was then lysed in water. A sample of erythrocytes before incubation was used to correct the K^+ content of incubated cells for the amount trapped in extracellular space. Cellular volume was quantified by the cyanomethemoglobin method of Drabkin and Austin (8). K^+ leakage was determined by flame emission at 766 nm (Varian Techtron model 1000). All experiments were done three times in duplicate.

Spectroscopic measurements. CD spectra were recorded with a Jobin-Yvon Mark V dichrograph, thermostated at 37°C. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^2 mol^{-1}). Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrophotometer.

Measurement of the relative proportions of monomeric, oligomeric water-soluble, and aggregated non-water-soluble AmB. The relative proportions of monomeric, oligomeric water-soluble, and aggregated non-water-soluble AmB were determined from 2 μM suspensions of AmB in PBS, obtained from the different stock solutions used in the kinetic studies. We took advantage of the differences in the absorption spectra of monomeric and self-associated (oligomers or aggregates of oligomers) AmB. For monomeric AmB, a peak is observed at 409 nm with ε = 120,000; for self-associated forms, there was a peak at 420 nm, with ε = 11,600. AmB at concentrations lower than 10^{-4} M dissolved in methanol is totally in the monomeric form and exhibits a peak at 407 nm, with ε = 150,000. CD was also used, since self-associated AmB presents an intense dichroic doublet centered at 340 nm (that is, positive and negative peaks at approximately 325 and 350 nm respectively), while the CD of monomeric AmB at this wavelength is negligible (3, 9, 13).

The suspensions were incubated for 5 min at 37°C. The suspensions were then divided in two samples. The first sample (sample a) was allowed to incubate for an additional 10 min at 37°C. CD and absorption spectra of this sample were then recorded. The second sample (sample b) was centrifuged at 12,000 × *g* for 10 min at 37°C. CD and absorption spectra of the supernatants, representative of the soluble forms of AmB were then recorded.

A_{407} of sample a, diluted 100-fold in methanol, gave the total concentration of AmB (A_t). A_{409} of the supernatant of sample b gave the concentration of monomeric AmB (A_m), as the contribution of self-associated AmB was considered negligible at this wavelength (less than 5%). A_{407} of sample b diluted 100-fold in methanol, gave A_m plus A_s (A_s is the concentration of water-soluble self-associated AmB). The concentration of non-water-soluble aggregated AmB (A_{ns}) was obtained from the following equation: $A_{ns} = A_t - (A_m + A_s)$. The results were corroborated with those obtained from CD measurements, which gave $A_{ns} + A_s$ and A_s.

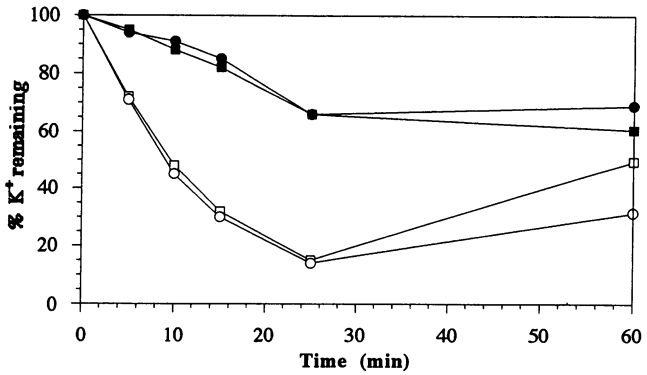

FIG. 1. Kinetics of AmB-induced release of K^+ from human erythrocytes, showing the influence of the concentrations of the stock solutions of AmB. Cell suspensions of 2×10^8 cells per ml and a temperature of 37°C were used. Symbols: ■, 4.2 mM AmB in DMF; □, 0.1 mM AmB in DMF; ●, 10 mM AmB in DMSO; ○, 0.1 mM AmB in DMSO.

RESULTS

AmB-induced K^+ leakage from erythrocytes. (i) Kinetics. Kinetics of K^+ release were recorded in the presence of 2 μM AmB. Four stock solutions were used: two stock solutions of AmB dissolved in DMF at concentrations of 4.2 and 0.1 mM AmB and two stock solutions of AmB dissolved in DMSO at concentrations of 10 and 0.1 mM AmB. AmB concentrations of 4.2 mM in DMF and 10 mM in DMSO corresponded to near saturation of these solutions. The final percentages of organic solvent in the four samples studied were 0.084, 2, 0.02, and 2, respectively. In order to take into account the possible effect of the total concentration of the organic solvent, two supplementary experiments in which solvent was added after injection of the more concentrated stock solutions were done in order to reach a final concentration of 2%.

From Fig. 1, it was observed that whatever the solvent, suspensions of AmB obtained from 0.1 mM stock solutions led to a much greater K^+ leakage than that found with suspensions of AmB obtained from the more concentrated stock solutions. The addition of solvent after the injection of the more concentrated stock solutions in order to obtain a final concentration of 2% with DMF stock solutions did not change the kinetics and with DMSO stock solutions led to a small increase in the rate of leakage after 15 min (data not shown). It is also worthwhile to note that partial recovery from the leak of K^+ was observed when diluted stock solutions were used.

(ii) Dose-response curves. The dose-response curves were obtained with the four stock solutions indicated previously, at 37°C with 2×10^8 cells per ml after a 60-min incubation. Figure 2 confirms that the sensitivity to AmB is higher when the stock solution is less concentrated. However, the effect is less drastic than the one observed for the initial kinetics of leakage. When the preincubation of AmB in PBS before the addition of erythrocytes was longer (2 h), the difference in toxicity between the various dispersions was reduced even more (data not shown).

CD spectra. Aqueous AmB suspensions were obtained as

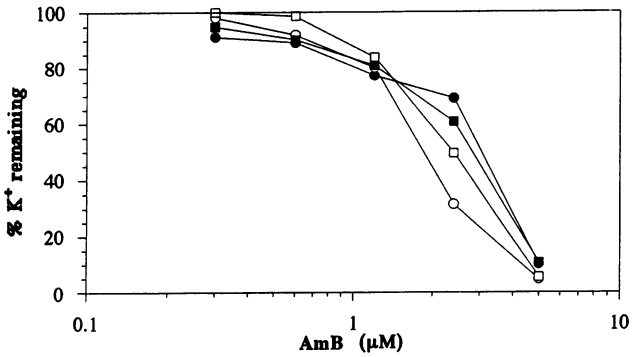

FIG. 2. Dose-response curves of AmB-induced release of K^+ from human erythrocytes, showing the influence of the concentrations of the stock solutions of AmB. Cell suspensions of 2×10^8 cells per ml, an incubation time of 60 min, and a temperature of 37°C were used. Symbols: ■, 4.2 mM AmB in DMF; □, 0.1 mM AmB in DMF; ●, 10 mM AmB in DMSO; ○, 0.1 mM AmB in DMSO.

usual by immediately diluting the desired aliquot of a DMSO or DMF stock solution in PBS at 37°C. Spectra were recorded 5 min after dilution. The CD spectra of 5 μM AmB are given in Fig. 3. This concentration is above the threshold of association of the antibiotic and, consequently, an intense dichroic doublet centered around 340 nm is observed (4). Δε increased with AmB concentration. A 20 to 30% decrease in Δε was observed after a 1-h incubation. The exact characteristics of the spectra were dependent on the nature of the solvent and the concentration of the stock solution. With DMSO, decrease of the concentration of the stock solution led to a shift of the dichroic doublet to blue. With DMF, decrease of the concentration of the stock solution led to a decrease in the intensity of the dichroic doublet without a wavelength shift.

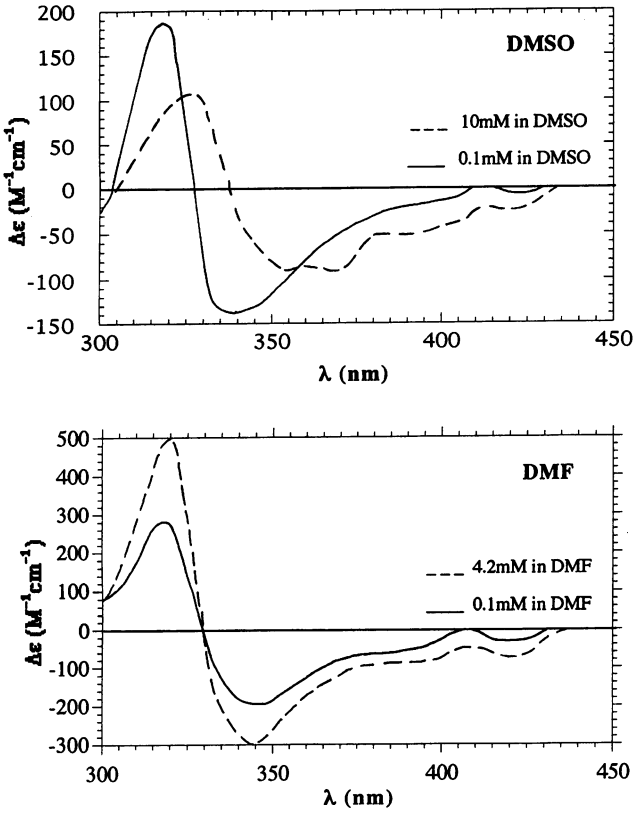

FIG. 3. CD spectra of 5 μM AmB, 5 min after dilution in PBS at 37°C, of the stock solution, showing the influence of the solvent and concentration of the stock solution.

Relative proportions of monomeric, oligomeric water-soluble, and aggregated non-water-soluble AmB. The results obtained with a 2 μM AmB suspension in PBS are shown in Fig. 4. The suspensions were prepared from the four stock solutions used in the kinetic studies. It appears that the concentration of soluble species is higher in suspensions from diluted stock solutions. For 0.1 mM stock solution in DMF, there is no self-associated non-water-soluble AmB present in the suspension. It was noticed that 2 h after dispersion of AmB in buffer at 37°C, an equilibrium was reached and the proportions of the three different forms of AmB were no longer influenced by the concentration of the antibiotic in the stock solution.

DISCUSSION

The interaction of an amphiphilic drug with the lipid part of the cellular membranes and the development of drug activity are complex. Indeed, depending on its concentration, the drug can be present in the aqueous medium in a soluble form (monomer or self-associated oligomer); beyond a critical concentration, the drug is in aggregated or micellar form. The drug can then have different types of activity (20). In some cases, only the micellar form can be active (12, 19) or bind to cell membranes (31). Binding of the monomeric or oligomeric form can lead to an active form either at once or after reorganization in micelles within the lipid bilayer, but only at concentrations exceeding the critical micellar concentration in the membrane (21, 24, 25, 27). Consequently, any factor modifying the equilibrium between the different forms present in the aqueous medium may change the overall activity of the drug. AmB affords a good example of such an interaction. The importance of the solubility and molecular spatial arrangement of AmB before its incorporation into membranes in determining the AmB toxicity to mammalian cells has already been stressed (10, 16).

The leakage of K^+ that is induced by AmB from erythrocytes has been studied several times as an index of polyene toxicity. In aqueous media, the antibiotic is present as an equilibrium of monomers, soluble self-associated oligomers, and nonsoluble aggregates of oligomers (22, 23, 28). In a previous study (3), we proposed that for AmB to induce K^+ leakage in cholesterol-containing membranes, it must be self-associated in water. A similar conclusion was drawn from a study on six AmB derivatives (18). Similarly, analysis of the basal urea permeability through model membranes (pure dimyristoyl phosphatidylcholine large unilamellar vesicles above their transition temperature) showed (7) that adding AmB at a concentration lower than 0.8 μM leads to a significant reduction in the permeability but adding AmB at a concentration higher than 0.8 μM, which corresponds to the threshold of AmB self-association in water, leads to an increase in permeability.

In the present study, by modulating the proportions of water-soluble and non-water-soluble AmB, we observed that the permeability to K^+ increases when the proportion of water-soluble AmB (monomers or self-associated oligomers) increases. Taking this and the previously published observations together lead us to the conclusion that actually only

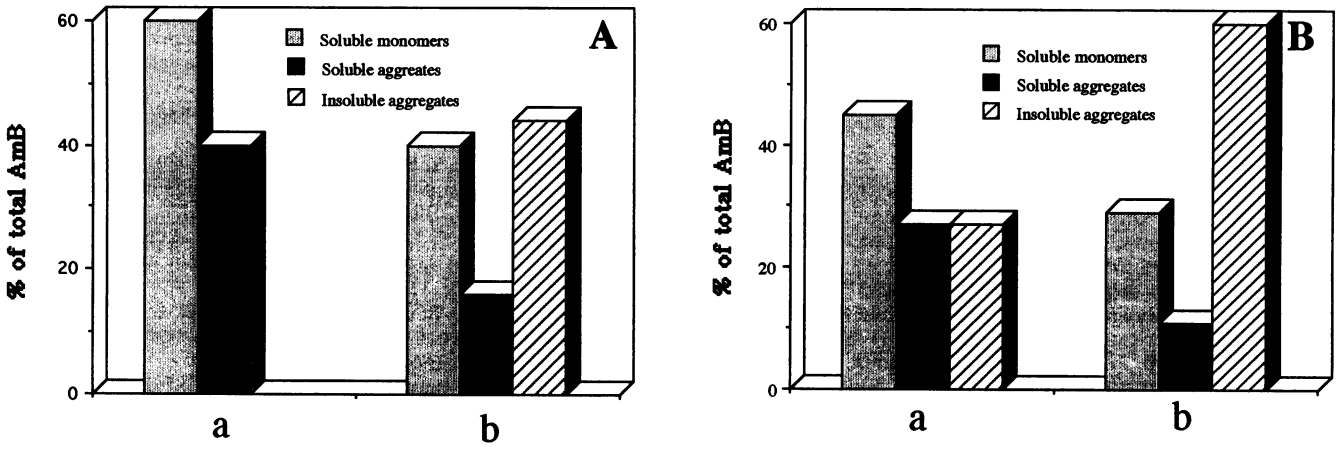

FIG. 4. Proportions of the different forms of AmB present in PBS, at a concentration of 2 μM, showing the influence of the solvent and concentration of AmB in the stock solution. Stock solutions using DMF (A) or DMSO (B) and the following different concentrations were used: 0.1 (a bars in both panels), 4.2 (b bars in panel A), and 10 (b bars in panel B).

water-soluble self-associated AmB is toxic, with the non-water-soluble form of AmB being much less toxic, if not entirely nontoxic. This conclusion is in agreement with the proposal of Gruda and Dussault (11), who hypothesized that the toxicity of antibiotic administered as monomers, with only traces of dimers, would be lower. Wietzerbin et al. (33), who studied AmB derivatives concluded that only solubilized species bind to erythrocytes and that disaggregation of aggregates is the limiting step in the binding process. However, in contrast with our conclusion, they hypothesized that the monomeric form of these derivatives is also active.

In the present study, we show, for the first time, the strong influence that the concentration of AmB in the organic solvent stock solution has on the kinetics of AmB-induced leakage of K^+ from erythrocytes: diluted stock solutions are much more toxic than concentrated ones. The lack of specification of the exact experimental conditions in other studies therefore makes comparing results difficult.

On the other hand, as far as the dose-response curves after 1-h incubation are concerned (Fig. 2), the influence of the concentration of the stock solution does not appear to be as drastic as for the kinetic measurements: 50% release of K^+ is obtained roughly between 1.8 and 3.2 μM AmB. There are two reasons for this weaker effect. The first is that the slope of the curve in the region of concentration at which activity begins to be observed is rather steep. Under these conditions, a small increase in the concentration of the active species induces a large increase in activity. The second reason is that we observed a partial restoration of K^+ intracellular levels after 1-h incubation with diluted stock solutions and a levelling off of the leakage with concentrated stock solutions. A similar effect was previously observed with rat hepatocytes (1). It has been attributed to a redistribution of AmB in the membrane over time, leading to the formation of inactive species and the cancelling of induction of permeability. It can also be assumed that the activity of the Na^+/K^+ ATPase, initially inhibited by the presence of AmB (32), was concomitantly restored and was sufficient to pump K^+ inside the cells.

It is interesting to note that our interpretation is also consistent with observations made with Fungizone. Fungizone is the clinically used form of AmB, solubilized by deoxycholate. This formulation consists of mixed AmB-deoxycholate aggregates coexisting with pure deoxycholate micelles. Although the AmB-deoxycholate aggregates are unstable at all concentrations, the rate of aggregation increases strongly with dilution (17). Below 5 μM the CD spectra of Fungizone are similar to those of AmB obtained from stock solutions in organic solvent, which indicates that AmB has dissociated from deoxycholate. Centrifugation and measurement of the decrease of the CD doublet indicates that the percent of AmB in non-water-soluble form is higher than with AmB obtained from stock solutions in organic solvents. In agreement with our hypothesis, the AmB dose giving 50% of K^+ release is shifted to 4.5 μM (data not shown).

The problem now is to understand how the structure of the association in the hydrophilic environment can have an effect on the structure of the AmB form inducing permeability to K^+ in the hydrophobic environment of the membrane. The present results can also shed light on the origin of the decrease in toxicity of AmB seen when AmB was incorporated in liposomes. On the one hand, we have shown here that only self-associated AmB in oligomeric water-soluble form is active and that the concentration of AmB dissolved in organic solvent determines the distribution of the different forms of AmB suspended in water. On the other hand, it has been shown that in vitro, the activity of liposomal AmB comes from the presence of free AmB that is not bound to liposomes (14, 15). However, it appeared in the same studies that in order to obtain an equivalent effect on erythrocytes, the quantity of "free" AmB of the liposomal formulations had to be higher than the quantity of AmB necessary when using stock solution in DMSO. From the present study, it can therefore be suggested that the role of the liposomes is to modify the distribution of the different species of "free" AmB, to the detriment of the monomeric and self-associated soluble forms. This result is not surprising, since these forms are the very species which will preferentially bind to liposomes during the preparation of liposomal AmB.

ACKNOWLEDGMENTS

This work was supported in part by grants from Institut Curie and Commission des Communautés Europeénnes (no. CI1-CT89-05).

We thank V. Joly for reading the manuscript and useful discussions.

REFERENCES

1. **Binet, A., and J. Bolard.** 1988. Recovery of hepatocytes from attack by the pore former amphotericin B. Biochem. J. **253:**435–444.
2. **Bolard, J.** 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta **864:**257–304.
3. **Bolard, J., P. Legrand, F. Heitz, and B. Cybulska.** 1991. One sided action of amphotericin B on cholesterol containing membranes is determined by its self association in the medium. Biochemistry **30:**5707–5715.
4. **Bolard, J., M. Seigneuret, and G. Boudet.** 1980. Interaction between phospholipid bilayer membranes and the polyene antibiotic amphotericin B. Biochim. Biophys. Acta **599:**280–293.
5. **Brajtburg, J., W. G. Powderly, G. S. Kobayashi, and G. Medoff.** 1990. Amphotericin B: delivery systems. Antimicrob. Agents Chemother. **34:**381–384.
6. **Chéron, M., B. Cybulska, J. Mazerski, J. Grzybowska, A. Czerwinski, and E. Borowski.** 1988. Quantitative structure-activity relationships in amphotericin B derivatives. Biochem. Pharmacol. **37:**827–836.
7. **Cohen, B. E.** 1992. A sequential mechanism for the formation of aqueous channels by amphotericin B in liposomes. The effect of sterols and phospholipid composition. Biochim. Biophys. Acta **1108:**49–58.
8. **Drabkin, D. L., and J. H. Austin.** 1932. Spectroscopy studies. I. Spectrophotometric constants for common hemoglobin derivatives in human, dog and rabbit blood. J. Biol. Chem. **98:**719–733.
9. **Ernst, C., J. Grange, H. Rinnert, G. Dupont, and J. Lematre.** 1981. Structure of amphotericin B aggregates as revealed by UV and CD spectroscopies. Biopolymers **20:**1575–1588.
10. **Ghielmetti, G., T. Bruzzese, C. Bianchi, and F. Recusani.** 1976. Relationship between acute toxicity in mice and polymorphic forms of polyene antibiotics. J. Pharm. Sci. **65:**177–183.
11. **Gruda, I., and N. Dussault.** 1987. Effect of aggregation state of amphotericin B and its interaction with ergosterol. Biochem. Cell Biol. **66:**177–183.
12. **Harnois, I., D. Genest, J. C. Brochon, and M. Ptak.** 1988. Micellization and interactions with phospholipid vesicles of the lipopeptide iturin A, as monitored by time-resolved fluorescence of a D-tyrosyl residue. Biopolymers **27:**1403–1413.
13. **Hemenger, R. P., T. Kaplan, and L. T. Gray.** 1983. Structure of amphotericin B aggregates based on calculations of optical spectra. Biopolymers **22:**911–918.
14. **Jullien, S., J. Brajtburg, and J. Bolard.** 1990. Affinity of amphotericin B for phosphatidylcholine vesicles as a determinant of the in vitro cellular toxicity of liposomal preparations. Biochim. Biophys. Acta **1201:**39–45.
15. **Jullien, S., A. Contrepois, J. E. Sligh, Y. Domart, P. Yeni, J. Brajtburg, G. Medoff, and J. Bolard.** 1989. Study of the effects of liposomal amphotericin B on *Candida albicans*, *Cryptococcus neoformans*, and erythrocytes by using small unilamellar vesicles prepared from saturated phospholipids. Antimicrob. Agents Chemother. **33:**345–349.
16. **Lamy-Freund, M. T., V. F. N. Ferreira, and S. Schreier.** 1989. Polydispersity of aggregates formed by the polyene antibiotic amphotericin B and deoxycholate. A spin label study. Biochim. Biophys. Acta **981:**207–212.
17. **Lamy-Freund, M. T., S. Schreier, R. M. Peitzch, and W. F. Reed.** 1991. Characterization and time dependence of amphotericin B: deoxycholate aggregation by quasielastic light scattering. J. Pharm. Sci. **80:**262–266.
18. **Legrand, P., A. Czerwinski, E. Borowski, and J. Bolard.** Unpublished data.
19. **Maget-Dana, R., I. Harnois, and M. Ptak.** 1989. Interactions of the lipopeptide antifungal iturin A with lipids in mixed monolayers. Biochim. Biophys. Acta **981:**309–314.
20. **Maher, P., and S. J. Singer.** 1984. Structural changes in membranes produced by the binding of small amphipathic molecules. Biochemistry **23:**232–240.
21. **Maurin, L., F. Baucel, P. Morin, and A. Bienvenue.** 1988. Interaction between a paramagnetic analogue of cholesterol and filipin. Biochim. Biophys. Acta **939:**102–110.
22. **Mazerski, J., J. Bolard, and E. Borowski.** 1982. Self association of some polyene macrolide antibiotics in aqueous media. Biochim. Biophys. Acta **719:**11–17.
23. **Mazerski, J., J. Grzybowska, and E. Borowski.** 1990. Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media. Eur. Biophys. J. **18:**1–6.
24. **Milhaud, J., M. A. Hartman, and J. Bolard.** 1989. Interaction of the polyene antibiotic amphotericin B with model membranes: differences between small and large unilamellar vesicles. Biochimie **71:**49–56.
25. **O'Neill, L. U., J. G. Miller, and N. O. Petersen.** 1986. Evidence for nystatin micelles in L-cell membranes from fluorescence photobleaching measurements of diffusion. Biochemistry **25:**177–181.
26. **Ramos, H., E. Romero, and B. E. Cohen.** 1988. The differential effect of liposomal amphotericin B on human erythrocytes and promastigotes of *Leishmania* sp. Acta Cient. Venez. **39:**135–139.
27. **Schwarz, G.** 1989. Thermodynamics and kinetics of incorporation into a membrane. Biochimie **71:**1–9.
28. **Strauss, G., and F. Kral.** 1982. Borate complexes of amphotericin B: polymeric species and aggregates in aqueous solutions. Biopolymers **21:**459–470.
29. **Szponarski, W., and J. Bolard.** 1987. Temperature-dependent modes for the binding of the polyene antibiotic amphotericin B to human erythrocyte membranes. A circular dichroism study. Biochim. Biophys. Acta **897:**229–237.
30. **Szponarski, W., J. Wietzerbin, E. Borowski, and C. M. Gary-Bobo.** 1988. Interaction of ^{14}C labelled amphotericin B derivatives with human erythrocytes: relationship between binding and induced K^+ leak. Biochim. Biophys. Acta **938:**97–106.
31. **Talbot, J. C., J. Lalanne, J. F. Faucon, and J. Dufourcq.** 1982. Effect of the state of association of melittin and phospholipids on their reciprocal binding. Biochim. Biophys. Acta **689:**106–112.
32. **Vertut-Doi, A., P. Hannaert, and J. Bolard.** 1988. The polyene antibiotic amphotericin B inhibits the Na^+/K^+ pump of human erythrocytes. Biochem. Biophys. Res. Commun. **157:**692–697.
33. **Wietzerbin, J., W. Szponarski, and C. M. Gary-Bobo.** 1990. Kinetic study of interaction between [^{14}C]amphotericin B derivatives and human erythrocytes: relationship between binding and induced K^+ leak. Biochim. Biophys. Acta **1026:**93–98.