Biochimica et Biophysica Acta, 1021 (1990) 39–45
Elsevier

BBAMEM 74701

Affinity of amphotericin B for phosphatidylcholine vesicles as a determinant of the in vitro cellular toxicity of liposomal preparations

Soizic Jullien [1], Janina Brajtburg [2] and Jacques Bolard [1]

[1] *Laboratoire de Physique et Chimie Biomoléculaire (U.A. CNRS 198), Tour 22, Université Pierre et Marie Curie, Paris (France)* and [2] *Department of Medicine, Division of Infectious Diseases, Washington University, School of Medicine, St Louis, MO (U.S.A.)*

(Received 27 June 1989)

Key words: Amphotericin B; Liposome; Vesicle; Polyene antibiotic; (*C. albicans*)

Candida albicans **and human erythrocytes were treated with liposomal amphotericin B (AmB) obtained by incubation of free AmB with small unilamellar vesicles (SUV) composed of unsaturated fatty acyl chains phosphatidylcholine (egg-yolk PC). Cellular effects were determined by changes in the K^+ internal content of cells and in the number of colonies formed by fungal cells or as hemolysis, measured as a decrease in haemoglobin retention by erythrocytes. Dose-response curves were obtained by two procedures: either the ratio of AmB to phospholipids was kept constant over the AmB concentration range used ($R = 10^{-2}$) or the phospholipid concentration was kept constant ($C = 0.2$ mM) and the concentration of AmB varied. The liposomal preparations of AmB were as active against fungi as AmB in dimethylsulfoxide but less active (internal K^+ changes) or inactive (hemolysis) against erythrocytes. On the other hand the binding of AmB to the SUV, as a function of the AmB concentration, was monitored by circular dichroism, fluorescence and UV absorption, in the two conditions used for the cellular studies. The amount of AmB bound when the total concentration of antibiotic was $2 \cdot 10^{-7}$ M was very low but increased with concentration and reached 90% at 10^{-5} M. In all the assays we used, the anticellular effects could be attributed to the levels of AmB remaining free (unbound to the lipids). The variations of these levels with total AmB concentration could therefore explain the increased selectivity of liposomal AmB in toxicity against fungi and erythrocytes as compared to that of AmB added as a solution in dimethylsulfoxide. Indeed fungal cells are sensitive to low concentrations of AmB in dimethylsulfoxide; at these concentrations, in liposomal preparations, AmB is not bound to phospholipids and therefore as active as if added in dimethylsulfoxide. By contrast erythrocytes are only sensitive to much higher concentrations of AmB in dimethylsulfoxide; at these concentrations AmB is almost totally bound to phospholipids and therefore much less active.**

Introduction

Amphotericin B (AmB) toxicity against fungal cells is greater than against mammalian cells. This selectivity of action can be increased in vivo by combining AmB with certain liposomal preparations (see Refs. 1 and 2, and references therein). Different studies have been performed in vitro to understand the origin of this effect and have shown that the toxicity of AmB for cultured mammalian cells is reduced by incorporation into liposomes while such is not the case for fungal cells [1–3]. It has been proposed that the rate and extent of AmB transfer between the various membranes (mammalian, fungal and liposomal) are important parameters which can be modulated by the lipid composition of the liposomes [1,2].

We have begun a systematic study of the relationship between the lipid composition of small unilamellar vesicles (SUV), their interactions with AmB (binding of AmB to SUV) and the in vitro anticellular effects of the liposomal AmB preparations made from them.

In the first part of this study [3], we used SUV made of phosphatidylcholines with saturated fatty acyl chains of different lengths (14:0, 16:0 and 18:0) and showed that the level of activity of the different liposomal preparations against erythrocytes and *Candida albicans*

Abbreviations: SUV, small unilamellar vesicles; MLV, multilamellar vesicles; AmB, amphotericin B; PC, phosphatidylcholine; DPPC, dipalmitoyl PC; DMSO, dimethylsulfoxide; CD, circular dichroism; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

Correspondence: J. Bolard, Laboratoire de Physique et Chimie Biomoléculaire (U.A. CNRS 198), Tour 22, Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris Cedex 05, France.

could be explained by the amount of AmB remaining unbound to the lipid in the vesicles as determined by circular dichroism [4]. Those preparations which bound the highest levels of AmB were the least active against cells.

Here we describe the second part of our study in which we measured binding of AmB to SUV prepared from egg-yolk phosphatidylcholine (EPC) containing unsaturated fatty acyl chains and the in vitro cellular toxicity obtained from the different preparations of liposomal AmB. As in the first part of this study we found that the in vitro cellular effects of the liposomal AmB preparations could be attributed to the activity of AmB remaining unbound in the preparations. The proportion of this free AmB depends both on the type of lecithin used in the SUV and on the initial concentration of AmB. For any particular AmB- liposomal preparation, the percentage of free AmB is higher when the total AmB concentration in the preparation is low, and lower when the initial concentration is high. Therefore, since AmB antifungal effects occur at lower AmB concentrations than the toxicity to mammalian cells, interaction of the antibiotic with phosphatidylcholine vesicles may decrease its effects on mammalian cells without affecting its antifungal activity.

Materials and Methods

The L-α-phosphatidylcholine tested, dipalmitoyl (DPPC) and egg-yolk PC, were purchased from Sigma and used without further purification. Amphotericin B was a gift from Squibb France.

K^+ effluxes were measured using a specific electrode, and recorded on a Servotrace. Absorption spectra were recorded on a Cary 219 spectrophotometer, dichroism spectra on a Jobin-Yvon Mark V dichrograph and fluorescence spectra on a Jobin-Yvon JY 3C spectrofluorimeter.

Preparation of SUV

Vesicles were prepared according Newman and Huang [5]. Briefly, vesicles suspensions were prepared by dissolving known amounts of phospholipid in chloroform in such a way that the total concentration of lipids remained constant. After removal of the solvent, 2 ml of phosphate buffer containing 10 mM phosphate and 150 mM NaCl, pH 7.4 (PBS) were added. The sample was sonicated above the transition temperature of pure phospholipid vesicles for 10 min (up to clarification of the suspension).

Preparation of liposomal amphotericin B

AmB dissolved in DMSO (10 mg/ml) was added to suspensions of SUV at the desired concentration and incubated for 1 h at room temperature. For the determination of the dose-response curves of liposomal AmB activity two procedures were used: either (i) the phospholipid concentration was kept constant ($C = 0.2$ mM) and the concentration of AmB was varied (procedure A) or (ii) the ratio of AmB to phospholipid was kept constant ($R = 10^{-2}$ or $R = 3 \cdot 10^{-4}$) over the entire AmB concentration range (procedure B).

In procedure B, aliquots of a stock-solution of AmB-SUV (2 mM in PC, and $2 \cdot 10^{-5}$ M in AmB for growth inhibition experiments and $6 \cdot 10^{-7}$ M in AmB for K^+ leakage experiments) were added to the cell suspension in order to have the desired concentration.

In our preparations AmB was added after the SUV had been formed. We chose this procedure rather than the addition of AmB during the preparation of SUV, in order to avoid the possible detruction of AmB by ultrasonication.

Preparation of erythrocytes

Human erythrocytes from normal male donors were isolated from blood anticoagulated with EDTA by centrifugation ($800 \times g$) for 10 minutes. The plasma and buffy coat were then removed and erythrocytes were washed three times with PBS before being dispersed in PBS. The cells were counted in a hematocytometer and used on the same day.

Preparation of fungal cells

Candida albicans strains B 311 were used. Cells were maintained on Sabouraud dextrose agar and transferred weekly. An inoculum from the culture was transferred to 10 ml of liquid Sabouraud medium, and incubated at 35° C. After 16 to 18 h incubation, 10 ml of fresh medium was added, and the mixture was incubated for an additional 1 h. Hematocytometer counts were done immediately before starting experiments.

For the determination of cell sensitivity to AmB: 1 ml of the cell suspension (10^8 erythrocytes/ml or $4 \cdot 10^7$ fungal cells/ml) was added to: (i) 1 ml of PBS containing AmB (DMSO), or (ii) the necessary amount of the stock-solution of liposomal AmB adjusted to 1 ml (procedure B), or (iii) 1 ml of the vesicles (0.4 mM) containing AmB (procedure A).

After a 1-h incubation the cells were centrifuged for 5 min at $800 \times g$, and washed three times with PBS:

(i) 500 μl of H_2O was added to the pellet of erythrocytes for lysis. K^+ was measured using a specific electrode and hemoglobin remaining in cells was estimated from the absorption at 560 nm.

(ii) 500 μl of PBS was added to the pellet of fungal cells. K^+ was measured after heating an aliquot 5 min at 100° C.

(iii) The pellet of fungal cells was diluted and 50 μl deposited on a Petri dish. Colony counts were done after 24 and 48 h. The numbers of colony-forming units (c.f.u.) were expressed in percentage of control value (incubation without AmB).

Binding of amphotericin B to EPC-SUV

(1) Procedure A

Binding was measured after a 1-h incubation of 0.1 mM EPC-SUV with various amounts of AmB in PBS, at 23° C.

(a) For the high concentrations in AmB ($> 5 \cdot 10^{-6}$ M) we used the procedure that we have previously described [4]. Briefly, circular dichroism spectra of these preparations were compared to those of free AmB incubated for the same time period in PBS. Free AmB was monitored by its CD measured at a wavelength at which AmB bound to SUV exhibited no signal.

In the concentration range studied (above 10^{-7} M), the CD spectrum of free AmB in PBS consists of an intense dichroic doublet centered near 340 nm, and negative bands at 368–393 and 423 nm.

The spectrum of AmB bound to EPC-SUV (AmB/lipid ratio $> 5 \cdot 10^{-3}$) consists of a dichroic doublet smaller than that of free AmB and centered near 328 nm, and weak positive bands.

When R, the molar ratio AmB/phospholipid, increased, we observed the appearance of the free AmB dichroic doublet, which superimposed on that of bound AmB.

(b) for AmB concentrations between $3 \cdot 10^{-7}$ M and $5 \cdot 10^{-6}$ M, the superposition of the two doublets rendered the results inaccurate. Therefore we monitored the binding of AmB by the fluorescence energy transfer from the fluorescent probe TMA-DPH, according to the procedure we described previously [6].

TMA-DPH is fluorescent when present in an hydrophobic medium, such as membranes. After adding AmB, we observed a decrease in the fluorescence intensity due to a non-radiative energy transfer. Energy transfer efficiency is a function of the surface density of energy acceptor (acceptors per phospholipid) and of the R_0 value for the donor-acceptor pair, where R_0 is the distance (in Å) between the donor and the acceptor at which the transfer efficiency is 50%. Using the results of Fung and Stryer [7], we can correlate the amount of energy transfer with AmB binding (AmB per phospholipid).

(c) At AmB concentrations between $2 \cdot 10^{-8}$ M and $3 \cdot 10^{-7}$ M we used the method of Witzke and Bittman [8]: that is the free monomeric AmB was monitored by its electronic absorption at 409 nm and compared to that of bound AmB at 414 nm.

(2) Procedure B (constant antibiotic / lipid ratio)

The concentrations used in this procedure were obtained by dilution of a stock-solution obtained by incubation of 10^{-5} M AmB and 1 mM lipid. The changes occurring when the suspension was diluted 100-, 500- and 1000-fold were monitored by circular dichroism (amplitude of the dichroic doublet) and electronic absorption at 409 nm (see above).

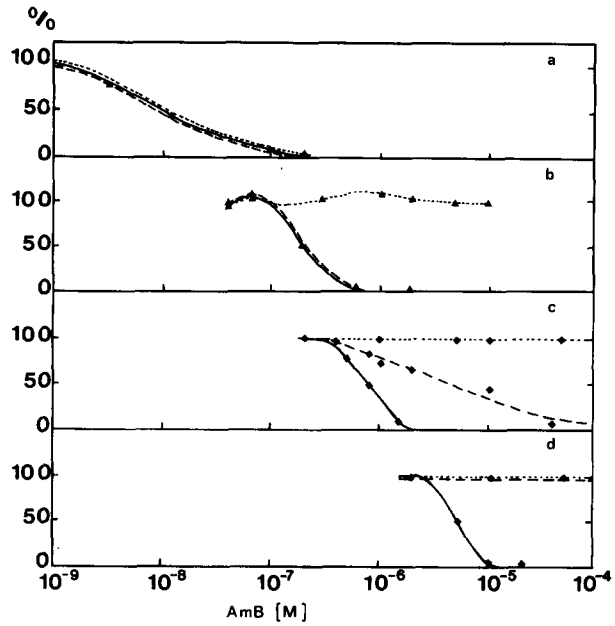

Fig. 1. The effects of free AmB or liposomal AmB on retention of K^+ by *Candida albicans* (a), viability of *Candida albicans* (b) and retention of K^+ (c) or hemoglobin (d) by erythrocytes. Cells were treated with AmB dissolved in DMSO (———) or liposomal AmB prepared from EPC SUV (- - - - - -). All preparations of liposomal AmB contained 0.2 mM of phospholipid. Points represent means ± S.D. from five experiments. For the purpose of comparison results obtained previously using liposomal AmB prepared from DPPC SUV [3] are also shown (· · · · · ·). The solid lines between experimental points are drawn solely to guide the eye. For the determination of C_{50}, the experimental data were analyzed according to the equation $E = E_0(1 - [C/(E + E_{50})])$ where E is the retention or viability for a dose C and E_0 is the retention or viability in the absence of AmB. These data were analyzed by a non-linear regression, using a Gauss-Newton algorithm.

Kinetics of dissociation were monitored by the increase of absorbance at 409 nm following the 1000-fold dilution of the stock solution: a 15-µl aliquot of stock-solution was rapidly mixed with 15 ml of PBS buffer at 23° C in a 5-cm path length cuvette.

Results

Cellular effects of liposomal amphotericin B

The effects of liposomal AmB prepared from EPC-SUV and of free AmB on *Candida albicans* and erythrocytes are shown in Fig. 1. The liposomal AmB preparations were obtained by incubating increasing concentrations of AmB with a constant concentration of lipids (0.2 mM) (procedure A). We also used liposomal AmB preparations in which the AmB-lipid molar ratio was kept constant (procedure B) with results identical to those shown in fig. 1.

The AmB-induced changes were measured as a decrease in retention of K^+ by *Candida albicans* cells (Fig. 1a), as a decrease in viability of *C. albicans* cells (ability to form colonies) (Fig. 1b), as a decrease in retention of

K^+ by erythrocytes (Fig. 1c) and as a decrease in retention of hemoglobin by erythrocytes (cell lysis; Fig. 1d).

Concentrations of AmB dissolved in DMSO (M) required for induction of 50% decreases in these indices were: $1 \cdot 10^{-8}$ (Fig. 1a), $2 \cdot 10^{-7}$ (Fig. 1b), $8 \cdot 10^{-7}$ (Fig. 1c) and $5 \cdot 10^{-6}$ (Fig. 1d). It is seen that liposomal (EPC) AmB was equally potent as AmB dissolved in DMSO in assays directed to *C. albicans*. When erythrocytes were the target, liposomal AmB was less potent than AmB dissolved in DMSO in inducing K^+ leakage and difference in activities were dependent on the extent of the induced damage: thus the induction of 20% decrease in retention of K^+ by erythrocytes required 5-times more of liposomal AmB than AmB dissolved in DMSO; for a decrease of 50%, 13-fold more liposomal AmB than AmB dissolved in DMSO was required and for 80% decrease 100-fold more. When we compared effects on a decrease in hemoglobin retention, it was noted that liposomal AmB was completely inactive up to concentration 10^{-1} M which is 12-times more than the concentration of AmB dissolved in DMSO required for 50% hemolysis.

Therefore, the activity of liposomal AmB from EPC-SUV was similar to that of free AmB in the lower range of AmB concentrations whereas in assays requiring higher concentrations of AmB the activity of liposomal AmB decreased. The observation that the activity of liposomal AmB in comparison to AmB dissolved in DMSO was variable in different assays was also made when liposomal AmB prepared from DPPC vesicles was used [3]. For the purpose of comparison these results are also plotted in Fig. 1. It is evident that liposomal AmB prepared from DPPC-SUV was as potent as AmB dissolved in DMSO (and as liposomal AmB prepared from EPC-SUV) in induction of K^+ leakage from *C. albicans*, the assay requiring the lowest AmB concentration. In the assay measuring the killing of *C. albicans*, which was conducted at the next highest level of AmB concentration, liposomal AmB prepared from EPC vesicles was as active as AmB dissolved in DMSO whereas the liposomal AmB from DPPC-SUV was inactive. The latter was also inactive in the two assays which required even higher AmB concentrations (leakage of K^+ from erythrocytes and lysis of erythrocytes).

Binding of amphotericin B to liposomes

To analyze the mechanism of liposomal activity, it is necessary to know the exact composition of the preparation, in particular the amount of AmB unbound to the SUV.

(a) Binding to EPC-SUV

In the determination of AmB-containing SUV activity we used two procedures. It was therefore necessary to determine the amount of AmB bound in both cases.

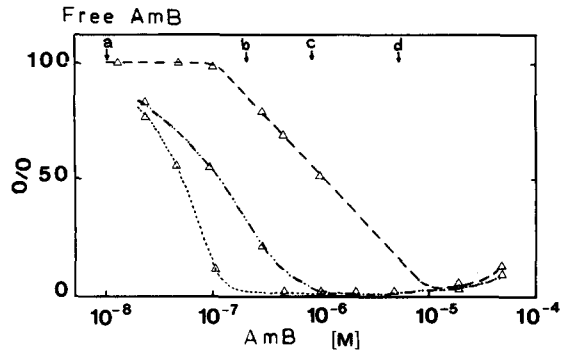

Fig. 2. Percentage of unbound AmB in liposomal AmB preparations as determined by electronic absorption ($2 \cdot 10^{-8}$ M–$3 \cdot 10^{-7}$ M), fluorescence energy transfer ($3 \cdot 10^{-7}$ M–$5 \cdot 10^{-6}$ M) and circular dichroism ($5 \cdot 10^{-6}$ M–10^{-4} M). Two procedures (A and B) were used for these preparations (see text). — — —, EPC SUV, procedures A and B; ··–··, DPPC SUV, procedure A; ·····, DPPC SUV, procedure B. Arrows indicate C_{50}, the concentrations of AmB dissolved in DMSO, necessary to induce 50% of (a) release of K^+ from *Candida albicans*, (b) viability of *Candida albicans*, (c) release of K^+ and (d) hemoglobin from erythrocytes.

(1) AmB binding to SUV at constant lipid concentration (0.1 mM) (procedure A). For AmB concentrations ranging from $4 \cdot 10^{-6}$ to $5 \cdot 10^{-5}$ M we determined the percentage of unbound AmB by CD. For lower AmB concentrations, that is between $4 \cdot 10^{-7}$ and $4 \cdot 10^{-6}$ M we used fluorescence, and for the lowest concentrations, electronic absorption. In the range of overlap, the various methods gave the same results.

For values of AmB concentrations below 10^{-7} M, the binding to the bilayer was zero. The percentage of bound AmB increased with AmB concentration and reached approximately 90% for $[AmB] = 10^{-5}$ M (Fig. 2).

(2) Amphotericin B binding to SUV at constant antibiotic/lipid ratio (procedure B). We determined that approximately 90% AmB was bound to the SUV in the stock-solution. The participation of unbound AmB to the absorption spectra was therefore small and in particular that of aggregated self-associated AmB negligible. It was then necessary to determine the changes occurring when the suspension was diluted. By successive dilution, 100-fold, 500-fold, 1000-fold, the specific dichroism of the suspension decreased while the electronic absorption at 409 nm, specific of free monomeric AmB increased (Fig. 3). These results indicate that the AmB-EPC complexation was destroyed by dilution. The amounts of AmB bound or free were determined from the amplitude of the dichroic doublet or the height of the absorption peak at 409 nm, 1 h after the dilution. They were strikingly similar to those obtained in procedure A.

In an other set of experiments ($R = 2.5 \cdot 10^{-2}$) the kinetics of dissociation after a 1000-fold dilution was monitored by the electronic absorption at 409 nm (Fig.

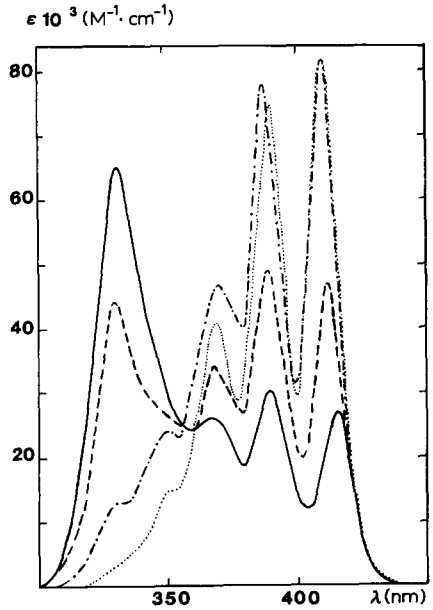

Fig. 3. Absorption spectra of a stock-solution of AmB (10^{-5} M) -EPC SUV (10^{-3} M) (———), diluted 10-fold (— — —), 100-fold (·–·–·). Absorption of AmB (10^{-7} M) dissolved in DMSO (·····). Incubations: 1 h at 20 °C.

4). Within 15 min after dilution the complex was almost totally dissociated. The dissociation kinetics could be fitted by the equation

$$A_t = A_{max} + A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

where: A_t was the absorption (409 nm) at time t, A_{max} was the absorption maximum obtained after dilution, k_1 and k_2 were rate constants and A_1 and A_2 the coefficients for each exponential (Table I).

(b) Binding of amphotericin B to DPPC-SUV

Using CD we already determined the binding of AmB to SUV at a constant lipid concentration for $5 \cdot 10^{-6}$ M < AmB < $5 \cdot 10^{-5}$ M and found it is around 90% [4]. For AmB concentrations lower than $5 \cdot 10^{-6}$ M, using fluorescence and electronic absorption as for EPC-SUV, we have now found that the amount of AmB bound remained higher than 80% even for AmB concentration as low as $3 \cdot 10^{-7}$ M (Fig. 2).

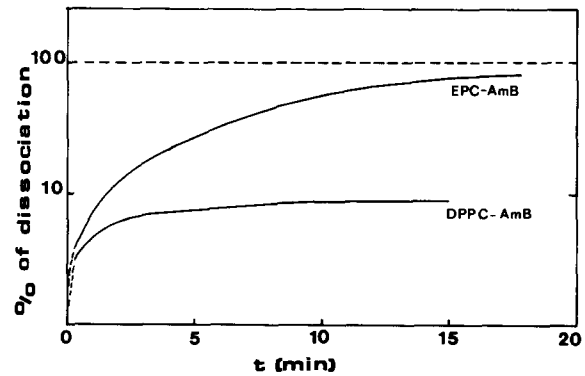

Fig. 4. Dissociation kinetics of a stock-solution of AmB ($5 \cdot 10^{-5}$ M) SUV ($2 \cdot 10^{-3}$ M). Trace of the absorption recording at 409 nm showing the increase of absorbance following the 1000-fold dilution of the stock-solution. The 100% corresponds to the absorbance of $5 \cdot 10^{-8}$ M AmB in buffer.

The determination of AmB remaining bound after dilution of a stock-solution of AmB-DPPC SUV ($R = 10^{-2}$), which corresponds to the procedure B was also determined as with AmB-EPC SUV. It appeared that in this case also the complex was destroyed by dilution but in the range $3 \cdot 10^{-8}$ to $5 \cdot 10^{-7}$ M in AmB, the amount of unbound AmB obtained was lower than that obtained by direct incubation. The kinetics of dissociation could be analyzed with parameters close to those found with AmB-EPC SUV (Table I).

Discussion

A number of studies have reported that AmB is more toxic to fungal than to mammalian cells. Liposomal AmB prepared from different lipid vesicles has been shown to be less active than AmB dissolved in DMSO against mammalian cells, whereas its activity against fungal cells has been shown to be the same as that of AmB dissolved in DMSO (for a review, see Ref. 10).

We have found, however, that this is not always the case. In our previous in vitro study [3], liposomal AmB was prepared with SUV composed of saturated phospholipids. Our preparations were less toxic to fungal cells than was AmB dissolved in dimethylsulfoxide.

In the current study we compared effects of free and liposomal AmB in inducing K^+ leakage from *C. albicans*

TABLE I

Dissociation kinetics of liposomal AmB (5 –10⁻⁵ M AmB, 2 –10⁻³ M lipid), obtained by 1000-fold dilution

The half-time of the reactions ($t_{1_{1/2}}$ and $t_{2_{1/2}}$) were calculated with the equation: $t_{1/2} = (\ln 2)/k$.

SUV	k_1 ($mol^{-1} \cdot min^{-1}$)	$t_{1_{1/2}}$ (min)	k_2 ($mol^{-1} \cdot min^{-1}$)	$t_{2_{1/2}}$ (min)	$\%A_{max(freeAmB)}$	N [a]
EPC	5.78 ± 1.60	0.119	0.286 ± 0.18	2.42	100 ± 14	13
DPPC	6.95 ± 3.19	0.100	0.234 ± 0.15	2.96	55 ± 10	6

[a] N = number of experiments

cells(a), in killing *C. albicans* cells(b), in inducing K^+ leakage from erythrocytes(c) and in lysing erythrocytes(d). The concentration of AmB dissolved in DMSO required for comparable decreases in these measured indices increases in the order a < b < c < d. In the assay requiring the lowest concentration of AmB dissolved in DMSO (a), AmB prepared from any phospholipid we have used (DPPC, DMPC, DSPC or PC) was as potent as AmB dissolved in DMSO. At the other extreme, in the assay requiring the highest AmB concentration (d), liposomal AmB obtained from DSPC vesicles was much less toxic than AmB dissolved in DMSO; liposomal AmB prepared from DPPC, DMPC or PC vesicles was completely inactive. In assays requiring the midrange concentration of AmB dissolved in DMSO (b and c) the potency of liposomal AmB depended on the lipid used.

We have found that the activities against *C. albicans* of AmB dissolved in DMSO and of AmB liposomal preparations (SUV) obtained with PC are identical. This result is in agreement with that obtained by Szoka et al. [2] on *Candida tropicalis* and *Saccharomyces cerevisiae*. It must be noted that these authors used preparations of liposomal AmB different from ours: SUV were prepared in the presence of AmB. As for the absence of activity (or the weak activity) of AmB-EPC vesicles on mammalian cells, our results on the inducement of hemoglobin release from human erythrocytes and the results of these authors on the cytotoxicity against murine macrophage cells RAW 264.7 and of Ramos et al. [9] on *Leishmania* cells are also in agreement.

In contrast, discrepancies appeared between our results on K^+ permeability inducement in erythrocytes and those obtained by Ramos et al. [9], which can be explained by the differences in incubation time. Juliano et al. [1] also obtained results on Rb^+ permeability inducement by AmB-MLV which can be considered at variance with ours: AmB-EPC MLV were not active in inducing $^{86}Rb^+$ release from erythrocytes, at least up to a concentration of 30 μM in AmB, while we observed a 50% release of K^+ for 6 μM AmB with AmB-EPC SUV. However it must be noted that these authors observed an activity with dioleoyl PC which, as EPC, is unsaturated.

In all the assays we used, the anticellular activities of liposomal AmB can be attributed to the activity of AmB remaining unbound in the preparation. In our preparations lipids were always in excess (minimum ratio AmB/lipid $R = 10^{-1}$); in these conditions the concentration of AmB remaining unbound depends on two factors (see below): the type of lipid used in the preparation of vesicles and the concentration of AmB added to these vesicles. Among lipids, the order of binding affinity to AmB is as follows: DPPC > DMPC > DSPC (for saturated lipids) > EPC (for saturated versus unsaturated lipids). As for the dependence of the percentage of bound AmB on the total concentration added, our results show that it decreases with the AmB concentration and is zero for 10^{-7} M in the presence of EPC SUV and for 10^{-8} M in the presence of DPPC SUV. Dilution from a pre-incubated stock-solution of liposomal AmB (procedure B) or incubation of SUV with the same amount of AmB (procedure A) gave the same amount of bound AmB when the liposomal preparations were made of EPC. This observation give information on the nature of the complex AmB-EPC SUV. Simple partitioning of AmB into bilayers does not occur because, in this case, the concentration of unbound AmB would remain more or less constant as the AmB/lipid ratio is changed by altering the AmB concentration while keeping the lipid concentration constant. Actually AmB binding to EPC SUV has the same characteristics as those observed with alamethicin [18] and filipin [19]: the antibiotic binds to the phospholipid membrane only when it is above a critical concentration. Furthermore in our experimental conditions we have seen that the lipid concentrations are always in large excess as compared to that of AmB which explains that, in both procedures, the extent of binding depends only on the antibiotic concentration.

The dilution of liposomal preparations obtained with DPPC SUV gave different results depending on the procedure used. This difference with EPC SUV is in agreement with the different nature of the interaction as demonstrated by circular dichroism [11].

Our results clearly indicate that an important factor determining in vitro, the cellular effects of liposomal preparations is the affinity of AmB for lipids used in these preparations. This notion can be extended to any system interfering in the interaction AmB–cell. A first example is given by serum proteins to which AmB binds [15]. Actually in the presence of lipoproteins the same observation is made as with AmB-EPC SUV: the effects of AmB on red blood cells are inhibited but not the effects on *C. albicans* [15]. Other examples can be obtained from other drug delivery systems such as sucrose monolaurate [13] or Intralipid® [14]. In contrast no difference in activity has been observed between Fungizone, the commercial form of AmB, and AmB dissolved in dimethylsulfoxide. Fungizone consists of mixed micelles obtained by addition of AmB to a deoxycholate suspension. The absence of modulation of deoxycholate, of the AmB activity, can be explained by the low affinity of the detergent for the drug, as demonstrated by the concentration dependence of the AmB CD [12] which shows that below 10^{-5} Fungizone is totally dissociated in AmB and deoxycholate.

The present study enabled us therefore, to explain the observation drawn from in vitro dose-effect relationships, that the in vitro selectivity of AmB between fungal cells and mammalian cells can be increased in the presence of liposomes or detergents. We give a physicochemical basis for the assumptions made by

Juliano et al. [1] and Szoka et al. [2] who interprete the increase in selective toxicity in terms of a selective transfer from donor liposomes to target cell membranes. Another observation that we made with DMPC or DPPC SUV [3], remains to be explained: at high doses of liposomal AmB (around 10^{-5} M in drug) no anticellular activity is observed while the amount of unbound AmB is such that it should be. The simplest explanation is that high doses of phospholipid liposomes or of AmB-containing liposomes have a protective effect against the action of unbound AmB, for instance by removing sterol from membranes [2]. We are currently testing this hypothesis.

The in vitro dose-effects relationships that we have been able to explain had been established in order to explain the effects observed in vivo. Actually the extrapolation to the in vivo situation is not staightforward. First AmB binds to serum proteins [15] and if liposomal AmB is given intravenously a rearrangement of the equilibrium between AmB and its vector will occur. For instance in serum, AmB-cholesterol-EPC SUV or AmB added as a solution in dimethylsulfoxide gives the same characteristic CD spectrum of AmB bound to lipoproteins [4]. Similarly with Fungizone a rapid dissociation of AmB from deoxycholate is observed [16]. On the other hand a delivery system for AmB may represent an improvement in vitro and not in vivo. Such is the case for instance of lipoproteins [17] or of sterol-containing dimyristoyl PC SUV [2]. It seems therefore that the origin of the in vivo increase of AmB therapeutic index, obtained by incorporation of the drug in liposomes, is still unclear. Perhaps can it be found in the protective effect of liposomes against the action of the drug. Studies are currently in progress to check this hypothesis.

Acknowledgments

This work was supported in part by a grant from Institut Curie and in part by grants AI 16228, AI 25903 and AI 72640 from Public Health Service U.S.A.

References

1 Juliano, R.L., Grant, C.W.M., Baber, K.R. and Kalp, M.A. (1987) Mol. Pharmacol. 31, 1–11.
2 Szoka, F.C., Milholland, D. and Barza, M. (1987) Antimicrob. Agents Chemother. 31, 421–429.
3 Jullien, S., Contrepois, A., Sligh, J.E., Domart, Y., Yéni, P., Brajtburg, J., Medoff, G. and Bolard, J. (1989) Antimicrob. Agents Chemother. 33, 345–349.
4 Jullien, S., Vertut-Croquin, A., Brajtburg, J. and Bolard, J. (1988) Anal. Biochem. 172, 197–202.
5 Newman, G.C. and Huang, C. (1975) Biochemistry 14, 3363–3367.
6 Henry-Toulmé, N., Séman, M. and Bolard, J. (1989) Biochim. Biophys. Acta 982, 245–252.
7 Fung, K.K.B. and Stryer, L. (1986) Biochim. Biophys. Acta 856, 11–18.
8 Witzke, N.M. and Bittman, R. (1984) Biochemistry 23, 1668–1674.
9 Ramos, H., Romero, E. and Cohen, B.E. (1988) Acta Cientif. Ven. 39, 135–139.
10 Swenson, C.E., Popexu, M.C. and Ginsberg, R.S. (1988) CRC Crit. Rev. Microbiol. 15, 51–531.
11 Bolard, J., Seigneuret, M. and Boudet, G. (1980) Biochim. Biophys. Acta 599, 280–293.
12 Ernst, C., Grange, J., Rinnert, H., Dupont, G. and Lematre, J. (1981) Biopolymers 20, 1575–1588.
13 Gruda, I., Gauthier, E., Elberg, S., Brajtburg, J. and Medoff, G. (1988) Biochem. Biophys. Res. Commun. 154, 954–958.
14 Kirsh, R., Goldstein, R., Tarloff, J., Parris, D., Hook, J., Hanna, N., Bugelski, P. and Poste, G.J. (1988) Infect. Dis. 158, 1065–1070.
15 Brajtburg, J., Elberg, S., Bolard, J., Kobayashi, G.S., Levy, R.A., Ostlund, R.E. and Medoff, G. (1984) J. Infect. Dis. 149, 986–997.
16 Lawrence, R.M., Hoeprich, P.D., Jagdis, F.A., Monji, N., Huston, A.C. and Schaffner, C.P. (1980) J. Antimicrob. Agents Chemother. 6, 241–248.
17 Koldin, M.H., Kobayashi, G.S., Brajtburg, J. and Medoff, G. (1985) Antimicrob. Agents Chemother. 28, 144–145.
18 Schwarz, G., Stankowski, S. and Rizzo, V. (1986) Biochim. Biophys. Acta 861, 141–151.
19 Milhaud, J., Mazerski, J. and Bolard, J. (1989) Biochim. Biophys. Acta 987, 193–198.