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Effect of polyoxyethyleneglycol (24) cholesterol on the solubility, toxicity and activity of amphotericin B

C. Tasset [1,*], F. Goethals [2], V. Préat [1,*] and M. Roland [1]

[1] *Laboratoire de Pharmacie Galénique, Université Catholique de Louvain, Brussels (Belgium)*
and [2] *Unité de Biochimie Toxicologique et Cancérologique, Université Catholique de Louvain, Brussels (Belgium)*

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Summary

Amphotericin B exhibits most of its toxic effects through its complexation with the cellular cholesterol. We study here the effect on this toxicity of the presence of a hydrosoluble cholesterol derivative (the polyoxyethyleneglycol (24) cholesterol) (PC) in the antibiotic formulation. We showed that PC could decrease the in vitro toxicity of Fungizone and of the borate derivative of the antibiotic: when present in specific proportions, PC was shown to be able to abolish the lytic effects of the drug on erythrocytes and isolated hepatocytes. We demonstrated that the optimal proportion of PC must be a compromise between a minimum quantity necessary to abolish amphotericin B toxicity and the proper toxicity of PC. PC is able to solubilize amphotericin B and the hydrosoluble formulation so obtained has a LD_{50} of 4.7 mg/kg compared with 2.7 mg/kg for Fungizone after i.v. injection in mice. When present in proportions leading to non-toxic amphotericin B formulations, PC did not modify the minimal inhibitory concentration of the antibiotic against *Candida albicans*. We conclude that PC can decrease the in vitro and in vivo toxicity of amphotericin B without modifying its in vitro antifungal activity.

Introduction

Amphotericin B remains the drug of choice for the treatment of systemic mycoses despite the severity of its side effects (anemia, renal damage and general malaise) and its poor water solubility.

The cytotoxic mechanism of this antibiotic is thought to be due to its ability to form membrane ion channels particularly in the presence of sterols (De Kruijff et al., 1974). Mammalian cells are less susceptible than fungal cells because of the higher affinity of amphotericin B for ergosterol (the predominant sterol in the fungal cell membrane) than for cholesterol (the predominant sterol in the mammalian cell membrane) (Readio and Bittman 1982). Despite this difference in affinity, the alteration of the mammalian cell membrane integrity can explain most of the cytotoxic effects of the drug.

Correspondence: C. Tasset, Laboratoire de Pharmacie Galénique, Université Catholique de Louvain, Brussels, Belgium.
* C. Tasset is "Aspirant" and V. Préat is "Chercheur qualifié" of the Fonds National de la Recherche Scientifique, Belgium.

On the basis of this theory, Janoff et al. (1985) described complexes between amphotericin B and a hydrosoluble cholesterol derivative and showed a lower toxicity with their combination. They explained this observation by a decrease of the affinity of the antibiotic for the cellular cholesterol without modification of the affinity for ergosterol.

The aim of this study was to analyze the influence of a hydrosoluble cholesterol derivative (the polyoxyethyleneglycol (24) cholesterol) (PC) on amphotericin B toxicity and activity. For that purpose, we elaborated a hydrosoluble complex of amphotericin B with PC and studied the toxicity and activity of this formulation. The influence of lower PC concentrations on the antibiotic toxicity and activity was studied using other hydrosoluble formulations of amphotericin B (Fungizone or the borate derivative of the antibiotic).

Materials and Methods

Amphotericin B (Am B) and Fungizone (a colloidal dispersion of amphotericin B in a micellar solution of deoxycholate) were obtained from Squibb. Deoxycholate was provided by Sigma. Solvents and borax were of analytical grade. Polyoxyethyleneglycol (24) cholesterol was a gift from Amerchol. The Sepacell filter was purchased from Baxter. Dulbecco's medium was obtained from Gibco and liquid Sabouraud medium from Difco.

Formulation

Complex amphotericin B-polyoxyethyleneglycol (24) cholesterol: Am B-PC. 17.2 mg Am B were dissolved in 20 ml dimethylformamide. 100 mg PC, dissolved in 20 ml methanol, were added to the solution which was evaporated to dryness at 60–65 °C under vacuum. The remaining film was suspended in 20 ml distilled water and a clear solution was obtained after simple shaking.

Complex amphotericin B-borate: Am Bor. A hydrosoluble borate derivative was prepared by the method previously described (Kral and Strauss, 1978). 100 mg Am B were dissolved in a 7 : 3 (v/v) mixture of dimethylsulfoxide and methanol saturated with borax. After sonication and filtration of the solution through a Whatman filter, the filtrate was precipitated in acetone. The precipitate was collected on a FH Millipore filter and vacuum-dried at 40 °C.

Complex Fungizone-polyoxyethyleneglycol (24) cholesterol: F-PC and amphotericin B-borate-polyoxyethyleneglycol (24) cholesterol: Am Bor-PC. The polyoxyethyleneglycol (24) cholesterol was added in different proportions to Fungizone or to the borate derivative dissolved in water.

For all the preparations including PC, the surface active agent proportion was expressed as a mol/mol (Am B/PC) ratio.

In vitro hemolysis

Blood from Wistar male rats (300 g) was collected on citrate and was filtered through a Sepacell filter after dilution in a phosphate buffer, pH 7.4 (PB). The red blood cells so obtained were washed three times in PB and were stored at 4 °C for less than a week. On the day of the study, the erythrocytes were washed three times. They were diluted in PB in such a way that the same red blood cell dilution in water gave an absorbance of 0.825 at 550 nm.

The cell suspension in PB was incubated at 37 °C for 3 h in a shaking bath with different Am B formulations at different concentrations.

The percentage of hemolysis was determined after centrifugation at 2000 rpm for 10 min, by measuring the absorbance of the supernatant at 550 nm and comparing it with the absorbance of the same red blood cell dilution in water (considered as 100% hemolysis).

LDH leakage from isolated hepatocytes

The hepatocytes were isolated by an enzymatic perfusion (Krack et al., 1980). They were suspended in a Dulbecco's medium (0.5×10^6 cells/ml) supplemented with 1.5% bovine serum albumin and were incubated with different Am B formulations (25 μg/ml or 21.5 μg/ml).

Plasma membrane integrity was evaluated at different times by measuring the leakage of the lactate dehydrogenase (LDH) into the incubation medium (Krack et al., 1980). The results are expressed as a ratio of released activity to the total activity.

Scanning electron microscopy

The hepatocytes were washed in PB (pH 7.4) after 30 min of incubation. They were fixed in 2.5% glutaraldehyde for 24 h at 4°C and then in 1% OsO_4 for 1 h at room temperature.

The cells were dehydrated through acetone, CO_2 critical point dried and coated with gold before examination in a Super mini-SEM scanning electron microscope.

Acute toxicity

NMRI male mice (25–30 g) were injected in the tail vein with different Am B preparations diluted in 5% glucose. Groups of 10 mice received different doses of each formulation. Mortality and body weight were followed for 2 weeks and the LD_{50} values were calculated.

In vitro antifungal activity

The minimal inhibitory concentration of the Am B preparations was determined by a tube dilution technique in a liquid Sabouraud medium.

A wild strain of *Candida albicans* (2.5×10^6/ml) was incubated with different Am B concentrations for 30 h at 29°C. The Am B preparations and the Am B powder were diluted in water and in dimethylsulfoxide, respectively, to give a concentration of 1 mg/ml. These stock solutions were then diluted in liquid Sabouraud medium to give final concentrations of 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195, 0.098 and 0.049 μg/ml. Tubes without drug were used as growth control.

The minimal inhibitory concentration is defined as the lowest drug concentration which inhibits clearly visible growth with a slight turbidity being ignored (Lennette et al., 1985).

Statistical analysis

Treatments on isolated hepatocytes were compared by using a two-way analysis of variance by the SAS procedure ANOVA ($P < 0.05$) (factors treatment-time).

Student's *t*-test was applied to determine the significance of the hemolytic activity of a treat-

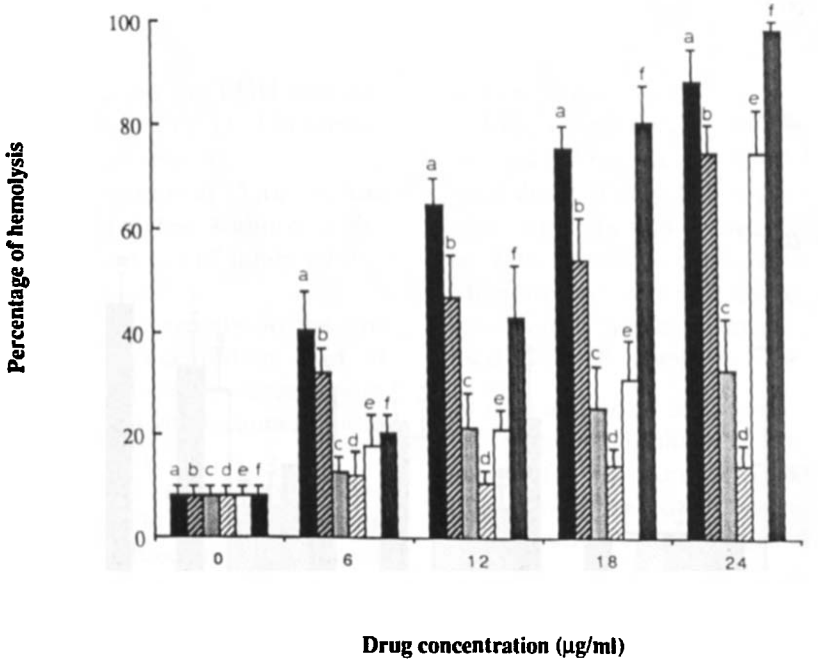

Fig. 1. Influence of Fungizone concentration and of Am B/PC proportion on the hemolysis: a, Fungizone; b, Fungizone-PC (1–0.07); c, Fungizone-PC (1–0.25); d, Fungizone-PC (1–0.33); e, Fungizone-PC (1–0.5); f, Fungizone-PC (1–0.66) ($n = 4$ except for Fungizone-PC (1–0.33) where $n = 5$).

ment. Differences were considered significant when they were below 5% probability.

Results

Hemolysis

Red blood cells were lysed by Fungizone and Am Bor (Figs. 1 and 2). Addition of PC to Fungizone progressively decreased the hemolytic activity of the antibiotic from the 1–0.07 to the 1–0.33 ratio, with the 1–0.33 proportion leading to a significantly non-toxic formulation. For higher PC proportions (1–0.5 to 1–0.66), hemolytic activity was again observed (Fig. 1).

Similarly, Am Bor toxicity was decreased by PC between the ratios 1–0.08 to 1–0.77, with the 1–0.29 and 1–0.39 ratios showing non-significant hemolytic activity (Fig. 2).

Deoxycholate was non-toxic at the concentration used in Fungizone (not shown) but PC showed some hemolytic activity (Fig. 3).

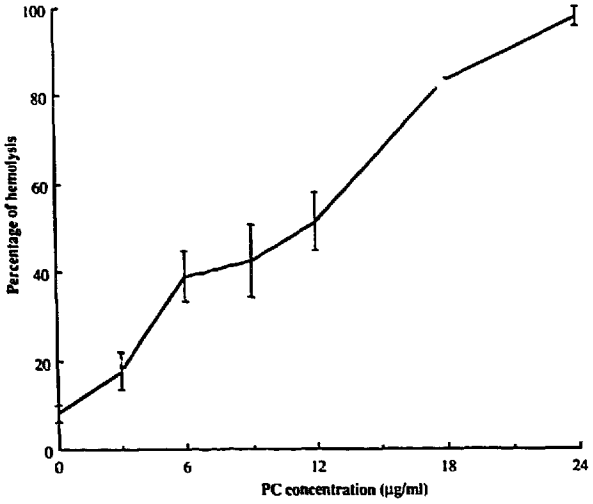

Fig. 3. Influence of PC concentration on hemolysis ($n = 3$).

Isolated hepatocytes: LDH leakage and scanning electron microscopy

Isolated hepatocytes incubated with Am Bor at a concentration of 21.5 µg/ml showed a rapid leakage of their LDH. Incorporation of PC in a 1–3.9 proportion significantly decreased the toxic

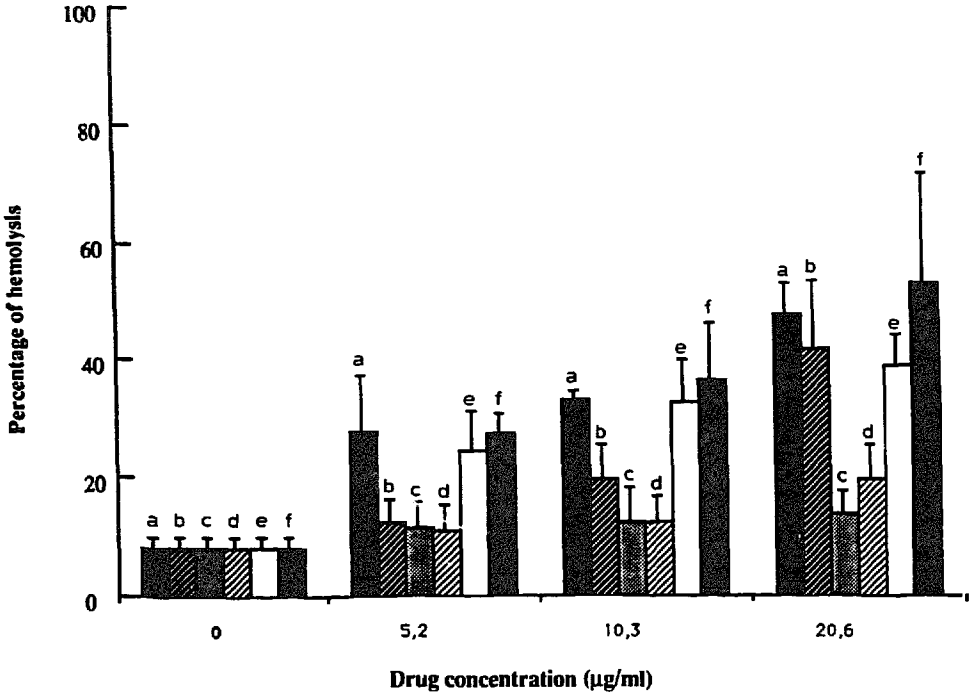

Fig. 2. Influence of the Am B borate derivative concentration and of Am B/PC proportion on hemolysis: a, Am Bor; b, Am Bor-PC (1–0.08); c, Am Bor-PC (1–0.29); d, Am Bor-PC (1–0.39); e, Am Bor-PC (1–0.58); f, Am Bor-PC (1–0.77) ($n = 3$).

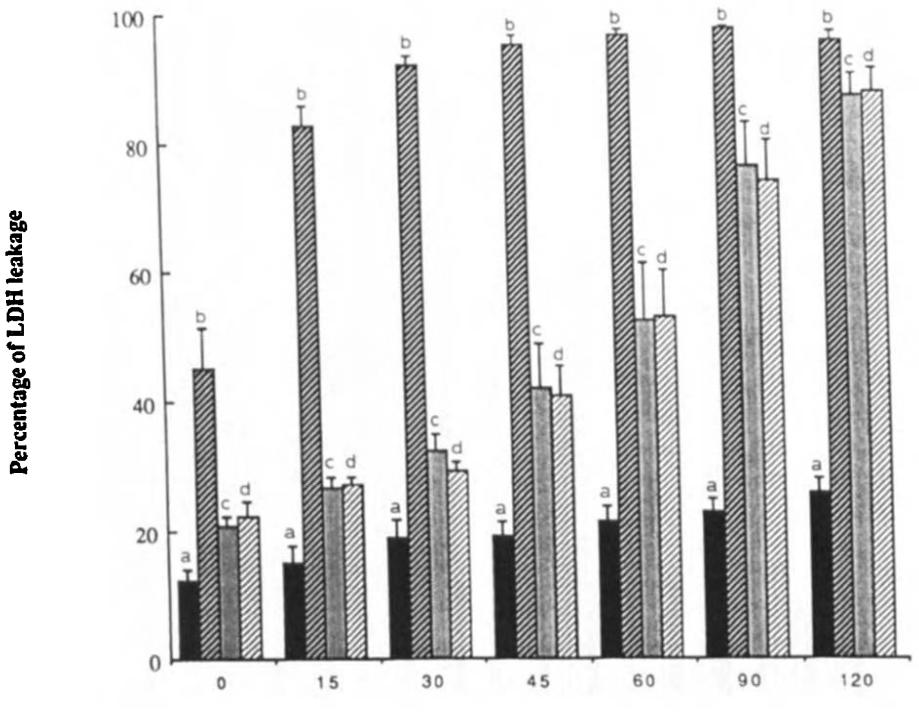

Fig. 4. Influence of Am B (21.5 μg/ml) formulations on the LDH leakage from isolated hepatocytes: a, control; b, Am Bor; c, Am Bor-PC (1 -3.9); d, Am B-PC (1–3.9) ($n = 3$ except for Am B-PC (1–3.9) where $n = 4$).

effect of Am Bor and delayed the LDH leakage. Am B-PC (1–3.9) and Am Bor-PC (1–3.9) formulations had similar toxicities (Fig. 4).

As shown in Fig. 5, Fungizone at 25 μg/ml was toxic for the isolated hepatocytes. Addition of PC between the ratios 1–0.5 and 1–1.67 inhibited this toxicity.

Deoxycholate had no lytic activity on the isolated hepatocytes at the concentration used in Fungizone (not shown), but cells incubated with more than 50 μg of PC/ml of medium showed leakage of their LDH (Fig. 6).

Fig. 7 shows important alterations of the hepatocyte membrane after 30 min of incubation with amphotericin B solubilised by PC and at a concentration of 21.5 μg of antibiotic/ml of medium. Under the same conditions, observation of cells treated by Fungizone was not possible because of cell lysis and at lower concentrations of antibiotic no similar alterations could be seen.

In vivo toxicity

LD_{50} values were 2.7 mg/kg for Fungizone but reached 4.7 mg/kg for Am B-PC (1–3.9) with the mice dying during the first two days after injection. Survivors showed decreased weights in parallel with increased doses during the first 4 days. Mice treated with PC alone showed no lethal toxicity nor weight decrease at the highest dose used in the PC complex.

In vitro antifungal activity

The minimal inhibitory concentration was 0.39 μg/ml for the Am B, Am Bor and Fungizone preparations tested. PC incorporation in proportions from 1–0.29 to 1–3.9 did not modify this value.

Discussion

In this study, it was shown that PC incorporation into different Am B formulations could de-

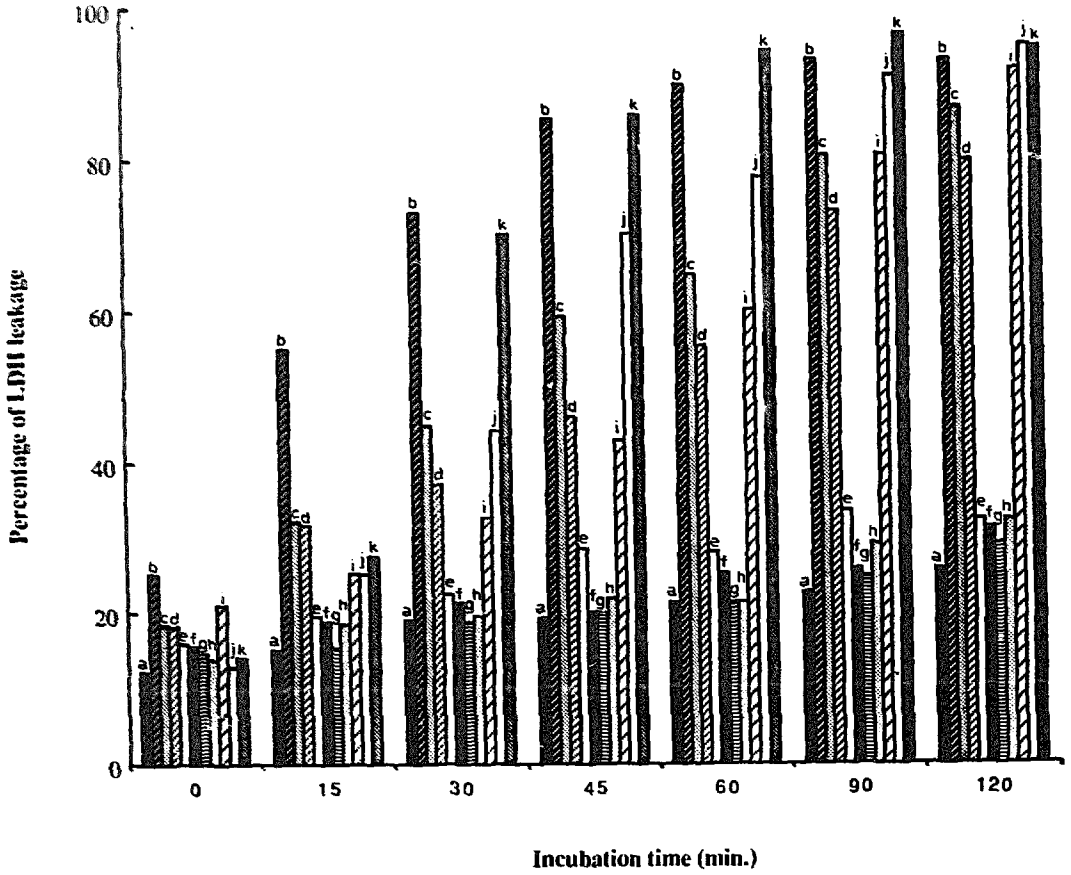

Fig. 5. Influence of PC on the Fungizone (25 μg/ml) induced LDH leakage from isolated hepatocytes: a, control; b, Fungizone; c, Fungizone-PC (1–0.25); d, Fungizone-PC (1–0.33); e, Fungizone-PC (1–0.5); f, Fungizone-PC (1–1); g, Fungizone-PC (1–1.33); h, Fungizone-PC (1–1.67); i, Fungizone-PC (1–3.33); j, Fungizone-PC (1–5); k, Fungizone-PC (1–6.67).

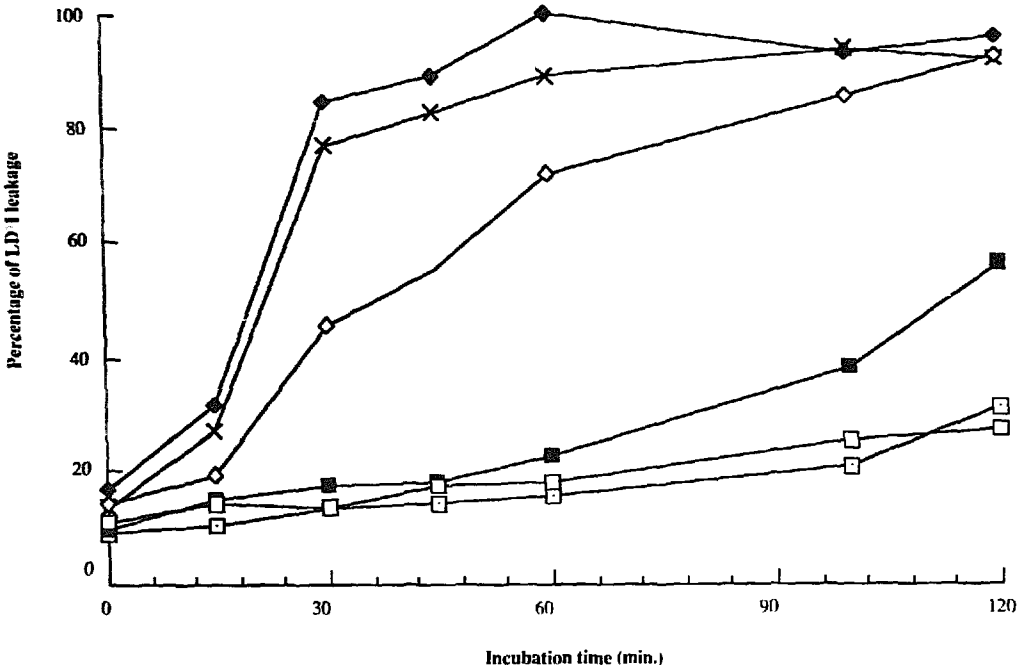

Fig. 6. Influence of PC concentration on LDH leakage from isolated hepatocytes incubated with 0 μg/ml (⊡); 50 μg/ml (□); 62.5 μg/ml (■); 125 μg/ml (◇); 187.5 μg/ml (×) or 250 μg of PC/ml (◆).

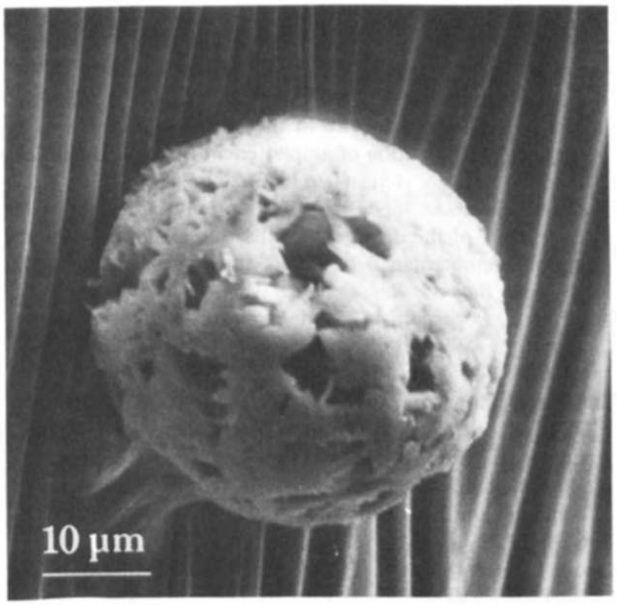

Fig. 7. Scanning electron micrograph of an hepatocyte incubated for 30 min in the presence of Am B-PC (1–3.9) at a concentration of 21.5 µg/ml.

crease the in vitro and in vivo antibiotic toxicity without affecting its in vitro antifungal activity.

The existence of an optimal Am B/PC proportion abolishing Am B toxicity on red blood cells and isolated hepatocytes was also demonstrated. It appears that this optimal ratio is independent from the Am B derivative and from the in vitro model of toxicity used: the optimal proportion on red blood cells is similar for Fungizone and Am Bor and is not different from the minimal optimal ratio abolishing Fungizone lytic activity on isolated hepatocytes.

For lower PC proportions, the antibiotic toxicity was not completely masked by PC and for higher PC proportions, the formulations were lytic because of the proper toxicity of PC. This PC toxicity was probably liable for the membrane alterations shown on the electron micrograph.

Therefore, the optimal proportion of PC seems to be the result of a compromise between a minimum quantity necessary and the surface active agent toxicity.

The surface active agent toxicity was also modified by Am B, PC alone lysing the erythrocytes at concentrations where the Fungizone–PC and the Am Bor–PC complexes were not hemolytic. This could be explained by a lower availability of this surface active agent to disrupt cellular membrane.

Improvement of Am B tolerance by PC could be due to: (1) a lower affinity of the antibiotic for the cellular cholesterol, (2) an enhancement in the water solubility of Am B or a specific aggregation state of Am B molecules in the formulations.

Janoff et al. (1985) studied the influence of a hydrosoluble cholesterol derivative on Am B toxicity and showed improvement of the antibiotic tolerance. They explained this by a lower affinity of the antibiotic for the cellular cholesterol. Using a similar cholesterol derivative, we observed the same decrease in toxicity but additionally demonstrated the importance of the Am B/PC proportion.

The decrease in the in vitro toxicity of Am B for mammalian cells was also observed with two hydrosoluble derivatives of the antibiotic (Malewicz et al., 1981). This effect was said to be due to an increase in the water solubility of these Am B derivatives. PC is also able to increase the Am B solubility and this could be an explanation of the lower toxicity of the Am B formulations including this surface active agent.

The aggregation state of Am B molecules seems to be important for the toxicity of the antibiotic. Recently, it was shown that the more aggregated state of the Am B molecules in lipidic structures reduces the toxicity of the antibiotic (Janoff et al., 1988). On the other hand, two surface active agents, sucrose monolaurate (Gruda et al., 1988) and Triton X-100 (Gruda et al., 1980), were shown to be able to decrease the aggregation state of Fungizone and Am B, respectively, with the former decreasing the Fungizone in vitro toxicity. Such an interaction could be expected with PC.

Sterols such as ergosterol and cholesterol, were shown to prevent the in vitro antifungal activity of polyene antibiotics (Gottlieb et al., 1958; Lampen et al., 1960). This action was explained by an interaction between the sterol and the antibiotic, reducing the effective antibiotic concentration still present (Lampen et al., 1960). Ergosterol and cholesterol decrease also the antifungal activity of the methylester derivative of Am B (Archer and Gale, 1970). However, in this study we did not

find any decrease in the Am B in vitro antifungal activity by the PC.

In conclusion, we demonstrated in this study the ability of PC to decrease the in vitro and in vivo Am B toxicity without affecting its in vitro antifungal activity. We also demonstrated the importance of the Am B/PC proportion to abolish the lytic activity of Am B. Several mechanisms could explain these observations. Further studies are now in progress to find the more appropriate one.

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