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# The Influence of Myrj 59 on the Solubility, Toxicity and Activity of Amphotericin B

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**Abstract**—The effect of Myrj 59 (a polyoxyethyleneglycol derivative of stearic acid) on amphotericin B (Am B) solubility, toxicity and activity has been investigated. We showed that Myrj 59 could solubilize the antibiotic. Moreover, it also decreased and abolished the haemolytic activity of the drug by increasing the resistance of the red blood cells and impairing the interaction of Am B with the cellular membrane cholesterol, but it did not modify the in-vitro antifungal activity of the drug. On the other hand, Myrj 59 did not decrease the acute in-vivo toxicity of the drug (LD50 and nephrotoxicity). In a previous study we have shown that a polyoxyethyleneglycol derivative of cholesterol could solubilize Am B and was able to decrease the in-vitro and in-vivo toxicity of the antibiotic without altering its in-vitro antifungal activity. The results of the present study suggest that the cholesterol moiety of the surfactant is not necessary to decrease the in-vitro lytic activity of the drug but could play a role in the reduction of the in-vivo toxicity.

Amphotericin B (Am B) remains a drug of choice in the treatment of systemic mycoses despite its poor water solubility and the severity of its side effects. Its mechanism of action is thought to be due to its ability to form membrane ion channels (associated with lethal permeability changes) particularly in the presence of sterols (De Kruijff & Demel 1974). The higher affinity of Am B for ergosterol (the predominant sterol in fungal cell membrane) than for cholesterol (the predominant sterol in mammalian cell membrane) (Readio & Bittman 1982) and the higher occurrence of channel formation at lower concentrations in the presence of ergosterol as opposed to cholesterol (Vertut-Croquin et al 1983) is a possible explanation for its clinical use.

Different attempts to decrease Am B toxicity include liposome encapsulation (New et al 1981; Taylor et al 1982; Lopez-Berestein et al 1983, 1987; Tremblay et al 1985; Kohno et al 1988; Hospenthal et al 1989; Patterson et al 1989) and an emulsion formulation (Kirsh et al 1988). The mechanism by which liposomes diminish Am B toxicity is not well understood. Juliano et al (1987) considered the reduction of toxicity exerted by liposomes to be the result of a selective transfer process regulated by the physical characteristics of donor and target cells, while Szoka et al (1987) suggested that the entrapment of Am B in liposomes slows its rate of transfer to the sensitive cellular target thereby reducing its toxicity.

Cholesterol incorporation in Am B formulations could decrease the toxicity of the antibiotic without altering its antifungal activity (Janoff et al 1985). In a study to verify this hypothesis, we have previously shown that a polyoxyethyleneglycol derivative of cholesterol could decrease the in-vitro and in-vivo toxicity of the drug without altering its in-vitro antifungal activity (Tasset et al 1990). The aim of the present work has been to study the effects of a polyoxyethyleneglycol derivative of stearic acid (Myrj 59) on the solubility, toxicity and activity of Am B.

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## Materials and Methods

### *Materials*

Amphotericin B (Am B) was obtained from Squibb for the nephrotoxicity study and from Sigma for the other experiments. Fungizone (a colloidal dispersion of Am B in a micellar solution of deoxycholate) was purchased from Squibb. Myrj 59 (polyoxyethyleneglycol (100) stearate) was a gift from Codibel. Ergosterol was from Aldrich and cholesterol from Sigma. The Sepacell filters were purchased from Baxter and the liquid Sabouraud medium from Difco. The kit for the detection of urea (using Berthelot's reaction) was obtained from Boehringer. The solvents and chemicals were of analytical grade.

### *Formulation of the complex Am B-Myrj 59 and of the complex Fungizone-Myrj 59*

Am B (20 mg) was dissolved in 20 mL dimethylformamide. An amount of Myrj 59 between 60 and 100 mg was added to the solution which was then roto-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 shaking. This preparation could be freeze dried and a clear solution reconstituted just before use upon addition of water to the dried product.

Myrj 59 was added in different proportions to Fungizone dissolved in water or in an isotonic phosphate buffer (pH 7·4).

For all the preparations containing Myrj 59, the proportion of surface active agent was expressed as a mol/mol (Am B-Myrj 59) ratio.

### *In-vitro haemolysis*

Blood from Wistar male rats, 250–300 g, was collected on citrate through an intracardiac puncture and was filtered

through a Sepacell filter after dilution in the phosphate buffer. The red blood cells so obtained were washed three times in the buffer and stored at 4°C for not more than a week. On the day of the study, the erythrocytes were washed three times in the buffer and were thereafter diluted in it in such a way that the same red blood cell dilution in water gave an absorbance of 0·825 at 550 nm (after centrifugation at 2000 rev $min^{-1}$ for 10 min).

To study the haemolysis induced by different Am B formulations, the antibiotic was dissolved in dimethylformamide (1 mg $mL^{-1}$). Fungizone, with or without Myrj 59, and the Am B-Myrj 59 complexes were dissolved in water at a concentration of 1 mg Am B $mL^{-1}$. These formulations were then diluted in the buffer and preincubated at 37°C for 15 min in a shaking bath. The red blood cells were then added to these Am B solutions and the incubation was continued at 37°C for 3 h. After incubation, the percentage haemolysis was determined by centrifuging the cell suspension at 2000 rev $min^{-1}$ for 10 min, the absorbance of the supernatant at 550 nm being compared with that of the same red blood cell dilution in water (considered as 100% haemolysis). The results were expressed as the percentage of haemolysis-±s.e.m.

To study the protection exerted by the surfactant against hypotonic haemolysis, different Myrj 59 concentrations were prepared in diluted buffer so that 60% of the red blood cells were haemolysed by hypotonicity. The Myrj 59 solutions were preincubated for 15 min at 37°C and the erythrocytes were then added. After incubation for 3 h in a shaking bath, the percentage haemolysis was determined as previously. The results were expressed as a relative percentage haemolysis ± s.e.m.

*Spectral analysis*

Fungizone and Myrj 59 were dissolved in the phosphate buffer. Ergosterol and cholesterol stock solutions were prepared in n-propanol at a concentration of $10^{-4}$ M. Solutions were prepared as follows: to Fungizone, n-propanol with or without sterol was added before the Myrj 59 solution. Final preparations contained $6{\cdot}5 \times 10^{-6}$ M Am B, 6·5% n-propanol, 0 or $6{\cdot}5 \times 10^{-6}$ M cholesterol or ergosterol and Myrj 59 concentrations from 0 to 0·5% (w/v). The solutions were mixed immediately after preparation and allowed to stand in the dark for 45 min (Gruda et al 1980). UV/visible spectra were then recorded using a double beam spectrophotometer. The interaction of Am B with the sterols was analysed by measuring the absorbance at the peak of the higher wavelength (Clejan & Bittman 1985).

*In-vitro antifungal activity*

The minimal inhibitory concentrations (defined as the lowest drug concentration inhibiting clearly visible growth, with slight turbidity being ignored (Lenette et al 1985)) of the Am B preparations were determined as described by Tasset et al (1990), who used a tube dilution technique in a liquid Sabouraud medium. Briefly, a wild strain of *Candida albicans* ($5 \times 10^6$ $mL^{-1}$) was incubated with various Am B concentrations of the different Am B formulations for 30 h at 30°C.

*LD50*

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 during 2 weeks and the LD50 values were calculated (Litchfield and Wilcoxon's method).

*Acute renal toxicity*

Six-day old Wistar rats received a single s.c. dose of Am B (20 mg $kg^{-1}$), as either Fungizone or Am B-Myrj 59 (1/0·71) or Fungizone-Myrj 59 (1/0·41)) diluted in 5% glucose. Such a treatment with Fungizone was previously shown to induce a rapid nephrotoxicity in young rats (Gray & Kavlock 1988). Twenty-four h after the injection, blood was collected following decapitation and urea serum concentrations were measured using the Berthelot reaction.

*Statistical analysis*

Student's *t*-test was applied to determine the significance of the haemolytic activity of each treatment and to compare the nephrotoxicity of the different Am B formulations.

## Results

*Haemolysis induced by different Am B formulations*

Under the conditions used, red blood cells were progressively lysed by Am B (6 to 24 μg $mL^{-1}$) (Tasset et al 1990). The complexation of Am B with Myrj 59 at the 1/1·02 and 1/0·61 molar ratios decreased the haemolytic activity of Am B (Fig. 1). Fungizone (Am B dissolved with sodium deoxycholate) was as haemolytic as Am B (dissolved in a minimum amount of dimethylformamide) for the red blood cells (Figs 1, 2). Addition of Myrj 59 to Fungizone to change the molar ratio from 1/0·05 to 1/0·41 decreased the haemolysis induced by the drug and the 1/0·41 molar ratio gave a significantly non-toxic formulation (Fig. 2).

Neither deoxycholate nor dimethylformamide were lytic for the erythrocytes at the concentrations used to solubilize Am B (data not shown). Myrj 59 was slightly haemolytic (< 20% haemolysis) between 60 and 120 μg $mL^{-1}$ (data not shown).

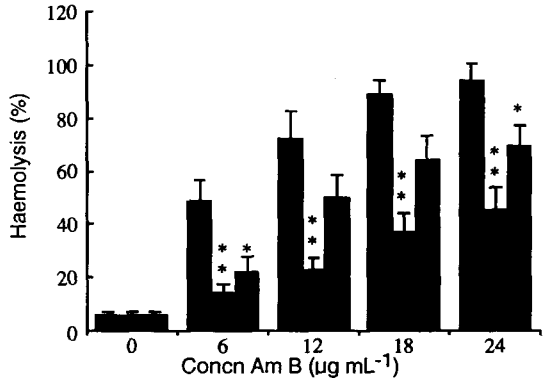


FIG. 1. Influence of Am B concentration and of Am B-Myrj 59 proportion on the haemolysis (n = 4). From left to right, AmB-Myrj 59: 1/0; 1/0·61; 1/1·02 (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$ vs Am B).

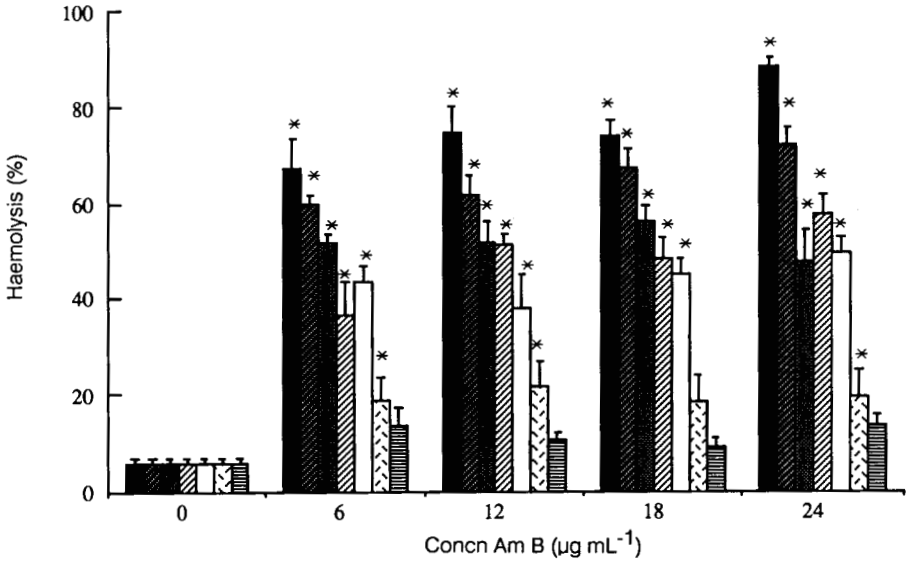


FIG. 2. Influence of Fungizone concentration and of Am B-Myrj 59 ratios on the haemolysis (n = 4 except for Fungizone-Myrj 59 (1/0·41) where n = 3). From left to right, Fungizone-Myrj 59: 1/0; 1/0·05; 1/0·1; 1/0·15; 1/0·21; 1/0·31; 1/0·41. (*$P < 0·05$ vs controls).

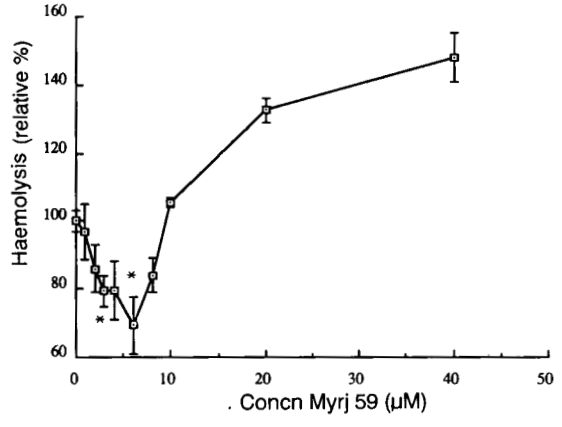


FIG. 3. Influence of Myrj 59 concentration on the haemolysis induced by hypotonicity. Results are expressed as a relative percentage of haemolysis. (n = 3) (*$P < 0·05$ vs control).

*Protection against hypotonicity*

To check whether the decrease in Am B-induced haemolysis by Myrj 59 was due to a protective effect of the surfactant on the red blood cell membrane, the effect of the surfactant on hypotonicity-induced haemolysis was measured.

Fig. 3 shows that when present at defined concentrations, Myrj 59 was able to protect the erythrocytes against hypotonic haemolysis. From 0 to $6 \times 10^{-6}$ M, Myrj 59 progressively decreased the haemolysis induced by hypotonicity. At a concentration of $6 \times 10^{-6}$ M, a maximal protection was observed with a relative percentage of haemolysis reaching 70%. For higher Myrj 59 concentrations this relative percentage increased over 100% because of Myrj 59 haemolytic activity.

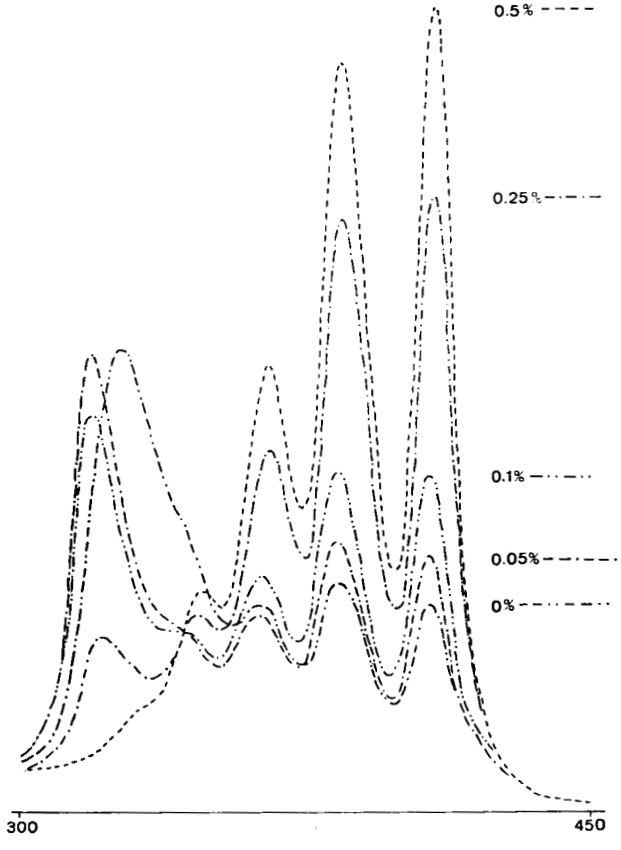


FIG. 4. Influence of Myrj 59 concentration on the UV/visible spectrum of Fungizone $6·5 \times 10^{-6}$ M in a phosphate buffer (pH = 7·4) containing 6·5% n-propanol.

*Spectral analysis*

Myrj 59 could also interfere with the interaction of Am B with the cellular cholesterol and in this way could protect the red blood cells against the antibiotic toxicity. To investigate this, we studied the influence of Myrj 59 on the complexation of Am B with cholesterol by using a UV/visible method. The UV/visible spectrum of Am B presents four bands between 300 and 450 nm and can be used to estimate the aggregation of the polyene molecules and their interaction with sterols (Bolard 1986).

When incorporated in the Fungizone solutions, Myrj 59 induced modifications in the UV/visible spectrum of Am B (Fig. 4). The absorbances at the higher wavelengths were increased whereas at the lower wavelength absorbance was decreased. These modifications are similar to those induced by ethanol (Rinnert et al 1977) and sucrose monolaurate (Gruda et al 1988) on the Am B spectrum and are thought to be due to a decrease in the aggregation state of the Am B molecules.

On the other hand, the association of Am B with sterols is accompanied by a decrease in the absorbance of the peak at the higher wavelength which represents the monomeric form of the antibiotic. This absorbance change can be used to determine the binding of the monomeric form of Am B with sterols (Clejan & Bittman 1985). The results of our study indicate that in 6·5% n-propanol and without Myrj 59, Am B (6·5 $\mu$g mL$^{-1}$) had a higher affinity for ergosterol than for cholesterol (Fig. 5). When surfactant concentration was increased, the Am B-cholesterol interaction decreased and at a concentration of 0·5% Myrj 59, no interaction with cholesterol was observed, whereas interaction with ergosterol was still present (Fig. 5), suggesting that Myrj 59 could increase the selectivity of the interaction of Am B with cholesterol and ergosterol.

*LD50*

Mice treated with the different Am B formulations showed, during the first four days after the injection, a decrease in their body weight parallel to the injected dose (data not shown). As indicated in Table 1, neither the complexation of Am B with Myrj 59 nor the addition of Myrj 59 to Fungizone was able to increase the LD50 values even though Myrj 59 did not cause weight decrease nor lethality at the higher concentration used in the formulations.

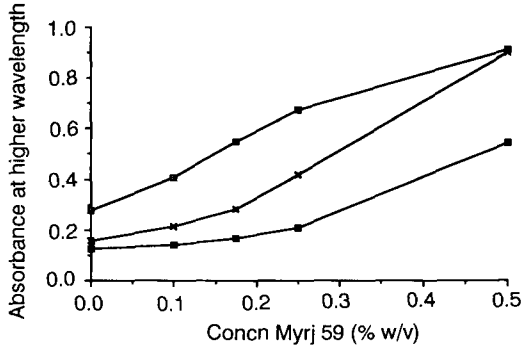


FIG. 5. Influence of Myrj 59 concentration on the absorbance at the peak of the higher wavelength of different Fungizone solutions ($6·5 \times 10^{-6}$ M) in a phosphate buffer containing 6·5% n-propanol. □ in the absence of sterol; x in the presence of cholesterol ($6·5 \times 10^{-6}$ M); ■ in the presence of ergosterol ($6·5 \times 10^{-6}$ M).

Table 1. LD50 of different Am B formulations injected intravenously to NMRI male mice.

| Formulations | LD50 values |
|---|---|
| Fungizone | 2·2 < 2·6 < 3·0 mg kg$^{-1}$ |
| Amphotericin B-Myrj 59 (1/0·61) | 2·0 < 2·2 < 2·4 mg kg$^{-1}$ |
| Fungizone-Myrj 59 (1/0·41) | 2·3 < 2·6 < 3·0 mg kg$^{-1}$ |

Table 2. Serum urea concentrations of Wistar rats injected s.c. with different Am B formulations (**$P < 0·01$ vs controls).

| Formulations | Serum urea mg/100 mL | s.e.m. | Number of treated animals |
|---|---|---|---|
| Controls | 48·4 | 1·6 | 14 |
| Fungizone | 101·4** | 4·7 | 14 |
| Fungizone-Myrj 59 (1/0·41) | 111·1** | 5·7 | 4 |
| Am B-Myrj 59 (1/0·71) | 110·2** | 7·6 | 4 |
| Myrj 59 | 46·9 | 2·9 | 4 |

*Renal toxicity*

Young rats treated with Fungizone showed an increase in serum urea concentrations compared with controls (Table 2) probably as a result of an increase in the tubular permeability of the nephrons leading to an increase in the urea reabsorption (Gray & Kavlock 1988).

The solubilization of Am B by Myrj 59 or the addition of Myrj 59 to Fungizone gave formulations as nephrotoxic as Fungizone in this model. However, neither deoxycholate nor Myrj 59 were nephrotoxic at the dose used in Fungizone and Am B-Myrj 59 (1/0·71).

*In-vitro antifungal activity*

The minimal inhibitory concentration was 0·39 $\mu$g mL$^{-1}$ for the Am B and Fungizone preparations tested. Myrj 59 incorporation at a molar ratio of (1/0·41) or (1/0·61) to Fungizone or Am B, respectively, did not modify this value.

## Discussion

It has been postulated that cholesterol incorporation in Am B formulations could decrease the toxicity of the antibiotic without altering its antifungal activity (Janoff et al 1985) but this remains to be confirmed.

In support of this, New et al (1981) found that cholesterol introduction in egg lecithin vesicles increased the LD50 of an Am B liposomal fomulation. Similarly, the presence of cholesterol in several small liposomes decreases the acute toxicity of the antibiotic formulations but their in-vitro antifungal activity was not modified (Szoka et al 1987). This protective effect exerted by the sterol was explained by a reduction of the interaction between the polyene and mammalian cell membrane because of the liposomal cholesterol (Tremblay et al 1985). Payne et al (1987) also demonstrated that the repeat dose toxicity of small liposomes could

be diminished substantially by the inclusion of ergosterol into the liposomal membranes.

On the other hand, it has been demonstrated that cholesterol (and also ergosterol) decreases the in-vitro antifungal activity of the drug entrapped in multilamellar vesicles (Hopfer et al 1984).

Our previous study had shown that a polyoxyethyleneglycol derivative of cholesterol was able to solubilize Am B in water (Tasset et al 1990). In the present work, it has been shown that Myrj 59, a polyoxyethyleneglycol derivative of a fatty acid, has the same property as the cholesterol derivative: similar concentrations of both surfactants are able to solubilize 1 mg Am B $mL^{-1}$ water. Therefore, we conclude that cholesterol in the surfactant structure is not necessary for solubilization of Am B in water. These results are not surprising since sodium deoxycholate (in Fungizone) and some Pluronics (Forster et al 1988), neither containing cholesterol in their structure, are also able to solubilize the antibiotic.

It was also shown that Myrj 59 could decrease and abolish the haemolytic activity of Am B. The solubilization of Am B with Myrj 59 (1/0·61 molar ratio) gave a significantly less lytic formulation than Am B (in dimethylformamide or as Fungizone). Moreover, the addition of Myrj 59 to Fungizone in an optimal concentration (1/0·41 molar ratio) abolished the lytic activity of the antibiotic.

A polyoxyethyleneglycol derivative of cholesterol can also decrease and abolish Am B haemolytic activity (Tasset et al 1990). One possible explanation of this effect was said to be the presence of cholesterol in the surfactant structure. The results obtained here with Myrj 59 clearly show that cholesterol is not necessary in the molecule of the surfactant to decrease or abolish Am B haemolytic activity. Therefore, cholesterol is not the only agent giving the protective effect to the cholesterol derivative.

On the other hand, Forster et al (1988), using Pluronic F 127 and L 92, obtained formulations as haemolytic as Fungizone suggesting either that not every surfactant is able to decrease the Am B membrane toxic effects or that the optimum surfactant concentration was not reached in these formulations.

Myrj 59 has surface-active properties and we investigated how this amphiphile could lead to less haemolytic Am B formulations. Myrj 59 can act on the cells themselves which are rendered more resistant to Am B lytic activity. The surfactant can also have an effect on the Am B molecules which could become less toxic for the red blood cells.

To check whether Myrj 59 protects the erythrocyte membrane from Am B toxicity, its effects on hypotonicity-induced haemolysis were measured. Amphiphiles are known to be able to haemolyse erythrocytes, but at lower concentrations they protect the red blood cells against hypotonic lysis or mechanically induced lysis. The mechanism of this antihaemolytic effect is not completely understood. One explanation could be that by intercalation into the lipid bilayer of the membrane the amphiphiles expand the membrane thereby permitting the cell to swell to a greater volume before it lyses (Isomaa et al 1986). In the present study, we have shown that Myrj 59 is able to protect the cells against hypotonic haemolysis with a maximal protection occurring at a concentration of $6 \times 10^{-6}$ M surfactant. This protection is not so important as that exerted against the lytic activity of Fungizone. However, the Myrj 59 optimum concentration showing the best protection against hypotonicity or Fungizone is within the same range.

The results of the UV/visible spectral analyses show that Myrj 59 is able to decrease the aggregation state of the Am B molecules. Moreover, at a concentration of 0·5% Myrj 59, the interaction of the antibiotic with cholesterol was not possible whereas that with ergosterol still occurred, suggesting that Myrj 59 could increase the selectivity of Am B for cholesterol and ergosterol.

Similarly, using sucrose monolaurate, Gruda et al (1988) showed that this surfactant could protect erythrocytes against Fungizone haemolytic toxicity whereas the antifungal activity of the Fungizone was not modified. Those authors correlated this with the increased selectivity of Am B complex formation with cholesterol and ergosterol in the presence of sucrose monolaurate. We found the same correlation. However, this study should be completed by using cellular membranes instead of isolated sterols because natural membrane sensitivity to Am B is also determined by membrane organization rather than a simple membrane component (Bolard et al 1980).

Even though Myrj 59 decreased the haemolytic activity of Am B, our formulations of Am B with Myrj 59 remained active against *Candida albicans*, as demonstrated by their minimal inhibitory concentrations.

Myrj 59 had a protective effect on Am B in-vitro haemolytic activity but was inactive against the acute in-vivo toxicity (LD50) of the polyene. This lack of correlation between in-vitro and in-vivo results has also been reported for several liposomal formulations of the drug (Szoka et al 1987). A polyoxyethyleneglycol derivative of cholesterol was able to increase the LD50 of Am B (Tasset et al 1990) but the conclusion that cholesterol plays a role in this protective effect is premature, other factors certainly being involved.

The major clinical problem with Fungizone is its nephrotoxicity. Our Am B formulations including Myrj 59 were as toxic as the Am B commercial preparation in young rats but a more relevant model is needed to determine human toxicity.

In conclusion, Myrj 59 was able to decrease and abolish the in-vitro haemolytic toxicity of Am B probably by increasing the selectivity of the interaction of the polyene with cholesterol and ergosterol and by increasing the resistance of the cells. Therefore, cholesterol was not, or not the only, causative agent of the protective effects shown with its polyoxyethyleneglycol derivative. However, Myrj 59 did not decrease the acute in-vivo toxicity of the antibiotic suggesting an eventual role of cholesterol in the protective effects exerted by the sterol derivative.

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