SELECTIVE TOXICITY OF THE POLYENE ANTIBIOTICS AND THEIR METHYL ESTER DERIVATIVES

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Summary:

The methyl ester hydrochlorides of amphotericin B and nystatin are less effective than the parent compounds in causing K^+ release from human erythrocytes. The parent compounds and the derivatives are of comparable activity toward Candida albicans. The enhanced selective toxicity of polyene methyl ester salts for C. albicans may mean that these antibiotics will be more effective therapeutic agents for systemic fungal infections.

Introduction:

Amphotericin B remains the only agent generally available for the treatment of systemic fungal infections in spite of the serious toxicity often associated with its use. Recently, the methyl ester hydrochlorides of amphotericin B and nystatin have been synthesized* (1). These acid salts of the methyl ester derivatives are water soluble and stable. AmBME retains antifungal activity but has a murine LD_{50} at least 15-25 times greater than AmB (2, 3). Lawrence and Hoeprich (4) have compared AmB with AmBME in the treatment of murine coccidioidomycosis. Although higher doses of AmBME were required for cure, the ester hydrochloride caused neither the azotemia nor the renal histopathology seen with AmB. Furthermore, treatment of a few patients with AmBME has not resulted in renal impairement (5).

Studies of the pharmacokinetics of AmB and the methyl ester salt in non-human primates reveal that they are handled somewhat differently (6).

* The following abbreviations will be used for the 4 polyenes referred to: amphotericin B, AmB; nystatin, Nys; amphotericin B methyl ester hydrochloride, AmBME; nystatin methyl ester hydrochloride, NysME.

Possibly the lower toxicity of the AmBME reflects its greater solubility and the lack of accumulation of the drug in target tissues such as the kidneys. Alternatively, AmB and AmBME may have different specificities for different biologic membranes. We have previously shown that Nys and AmB do indeed have different specificities for several natural and model membrane systems (7-9).

Using a modification of the method described by workers at the Rutgers Institute of Microbiology (1), we have prepared the methyl ester hydrochlorides of AmB and Nys. The derivatives retain excellent antifungal activity. To determine the relative activities of the 4 compounds on fungal versus mammalian cell membranes, K^+ release from C. albicans and human erythrocytes was determined using a potassium-specific electrode.

Materials and Methods:

AmB and Nys were obtained from the Squibb Institute for Medical Research, New Brunswick, New Jersey. The methyl ester hydrochlorides were prepared following the procedure of Mechlinski and Schaffner (1). The methyl esters, prepared by reaction of polyene with diazomethane, were checked for purity by thin layer chromatography and when no detectable parent polyene remained, they were converted into the hydrochloride salts. The final preparations of hydrochlorides were completely soluble in water and gave one major spot by thin layer chromatography. Stock solutions of drugs were made in dimethyl sulfoxide and stored at -15°C for a maximum of 3 days.

Human erythrocytes were obtained from freshly-drawn, heparinized blood. The cells were washed by centrifugation 3 times with cold 0.15M saline and then suspended in sodium phosphate (10mM phosphate, pH 6.8)-buffered 0.15M saline containing 27mM sucrose (buffered saline-sucrose) at a concentration so that 2 ml of the suspension gave an optical density of 1.0 at 540 nm after hemolysis with digitonin (10µg/ml). The suspension was kept on ice until used within 2 hours.

A clinical isolate of C. albicans was grown at 30°C with agitation in a medium containing 1.0% tryptone, 0.5% yeast extract, 0.5% glucose and 0.5% sodium chloride, pH adjusted to 7.0 with 1N sodium hydroxide. Cells in the logarithmic phase of growth were harvested and washed by centrifugation 3 times with cold buffered saline-sucrose. The cells were suspended in this medium at a concentration of about 1.5×10^8 cells/ml for K^+ release experiments.

To determine the minimum inhibitory concentration of the antibiotics, serial 2 fold drug dilutions in growth medium were inoculated with approximately 1×10^3 C. albicans/ml. The tubes were incubated at 30°C for 18 hours and then checked visually for growth. The lowest concentration of drug yielding no visible growth was identified as the minimum inhibitory concentration.

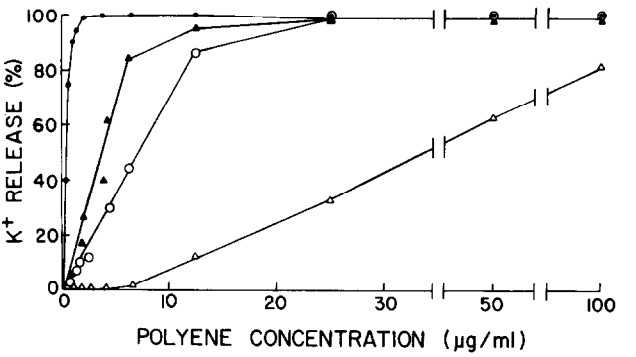

Figure 1: Dose-response curve for K^+ release from erythrocytes as a function of polyene concentration. Details are described in the text. Results with the different polyenes are represented by the following symbols: AmB (●), AmBME (▲), Nys (○), NysME (△).

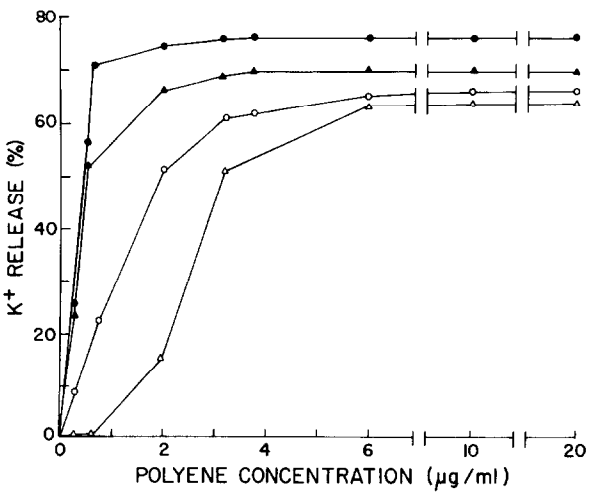

Figure 2: Dose-response curve for K^+ release from C. albicans as a function of polyene concentration. Symbols for the polyenes are as used in figure 1.

The K^+ concentration was determined using a K-specific liquid membrane electrode (Orion 93-series, Orion Research, Cambridge, Massachusetts). To determine the release of K^+ at various drug concentrations, 2 ml of cell suspension (C. albicans or erythrocytes in buffered saline-sucrose) were distributed into tubes containing 0.1 ml of dimethyl sulfoxide containing different amounts of drugs. The controls contained dimethyl sulfoxide without any drug. The tubes were incubated at 20°C for 30 min. The K^+ released was then determined. For kinetic studies, the electrode was dipped into a cell suspension and the K^+ level determined just before

TABLE 1: Ratio of polyene concentrations (μg/ml) causing 50% K^+ release from erythrocytes and C. albicans

POLYENE	ERYTHROCYTES/C. ALBICANS
AmB	0.50/0.45 = 1.1
AmBME	3.75/0.50 = 7.5
Nys	7.25/2.0 = 3.6
NysME	39.0/3.20 = 12.3

adding the drug. The drug was then added, to give a final concentration of 50μg/ml for AmB or AmBME and 100μg/ml for Nys or NysME. The suspension was stirred magnetically throughout the experiment, and K^+ concentration determined at intervals.

The amount of K^+ released is expressed as percent of the total K^+ release and this is calculated from the expression:

$$\frac{K^+ \text{ release in presence of drug} - \text{blank control}}{\text{total amount of } K^+ - \text{blank control}} \times 100$$

The total amount of K^+ was determined by adding digitonin (10μg/ml) to erythrocytes or by heating C. albicans in a boiling water bath for 2 min.

Results:

The minimal inhibitory concentrations for C. albicans of AmB, AmBME, Nys and NysME, respectively, were 0.625, 0.625, 12.5 and 25μg/ml.

In figure 1, the dose-response curves for K^+ release from erythrocytes for the 4 polyenes are plotted. The concentrations of antibiotics causing 50% K^+ release in 30 min. for AmB, AmBME, Nys, and NysME, respectively, were 0.50, 3.75, 7.25 and 39.0μg/ml.

Similar data for C. albicans are presented in figure 2. The concentrations of antibiotics causing 50% K^+ release in 30 min. for AmB, AmBME, Nys and NysME, respectively, were 0.45, 0.50, 2.0 and 3.20μg/ml.

Table 1 shows the ratios of polyene concentrations causing 50% K^+ release from erythrocytes divided by that required for C. albicans. The ratio for AmB is 1.1 reflecting a quite similar K^+-releasing potency for erythrocytes and C. albicans. The 3 other polyenes are more effective

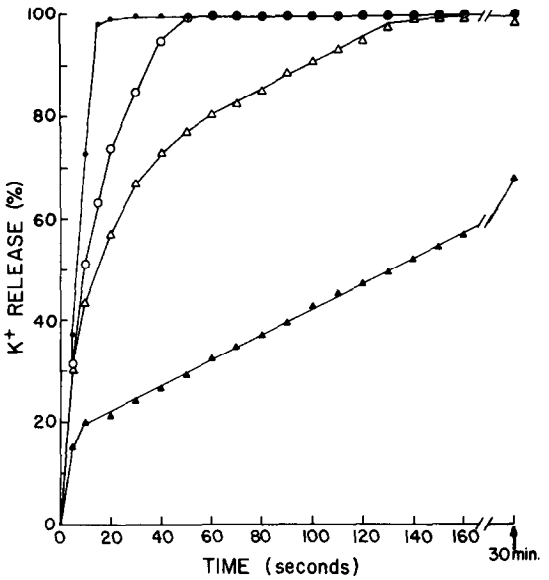

Figure 3: Rate of K^+ release from erythrocytes caused by the 4 polyenes. Symbols for the polyenes are as used in figure 1. Details are described in the text.

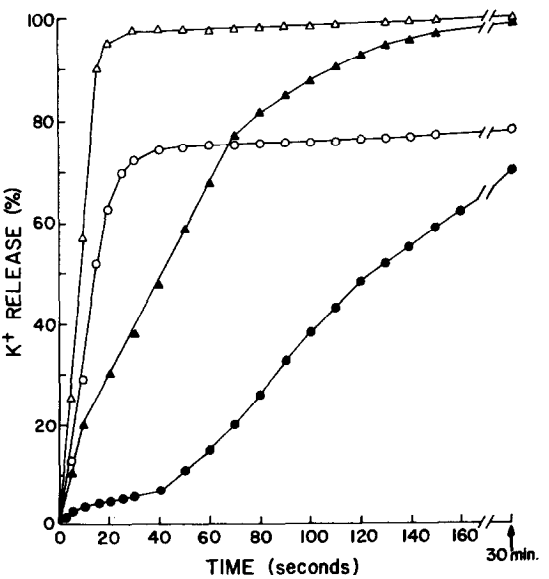

Figure 4: Rate of K^+ release from C. albicans caused by the 4 polyenes. Symbols for the polyenes are as used in figure 1. Details are described in the text.

TABLE 2: Ratio of times (seconds) required for 50% K^+ release from erythrocytes and C. albicans

POLYENE	ERTYHROCYTES/C. ALBICANS
AmB	7/125 = .05
AmBME	141/41 = 3:43
Nys	10/15 = .67
NysME	15/8 = 1.88

toward C. albicans than for erythrocytes. AmBME has about 7 fold greater selectivity than AmB for the fungi based on these dose-response curves (7.5/1.1). NysME shows the greatest selectivity - more than 11 times that of AmB (12.3/1.1), but it is also the least potent agent on a weight basis versus C. albicans.

In the next 2 figures, the rates of K^+ release from erythrocytes (figure 3) and C. albicans (figure 4) induced by the 4 polyenes are plotted. The concentrations of AmB and AmBME were 50µg/ml; of Nys and NysME, 100µg/ml. The time required to yield 50% K^+ release from erythrocytes were as follows: AmB 7, AmBME 141, Nys 10 and NysME 15 seconds. For C. albicans the times were: AmB 125, AmBME 41, Nys 15 and NysME 8 seconds.

In Table 2, the ratios (erythrocytes/C. albicans) of the times required by each drug to release 50% of the K^+ are presented. AmB works more quickly on erythrocytes than C. albicans; the opposite is true with AmBME. When these data are used as measure of selectivity of drug action, AmBME is about 68 times more selective than AmB (3.43/.05). The comparable figure for NysME and Nys is about 2.8 fold (1.88/.67).

Discussion:

The development of an effective antifungal agent with less toxicity for man than AmB is essential. Based on experiments with liposome model membrane systems we have demonstrated previously that Nys has a greater

selectivity for ergosterol-containing than cholesterol-containing membranes as compared with AmB (7-9). The data presented here with C. albicans (in which ergosterol is the principal membrane sterol) and erythrocytes (in which cholesterol is the principal membrane sterol) support this observation. Unfortunately, Nys is less potent than AmB and, hence, may not be effective antifungal therapy.

For reasons that are not clear, AmBME has a markedly more selective activity than AmB for C. albicans than for erythrocytes. The minimal inhibitory concentrations of the 2 agents for C. albicans are equal. Furthermore, the enhanced water solubility of AmBME will likely make it much easier to administer to patients than AmB and possibly improve the distribution of the drug in the body.

Sucrose was used in these experiments to protect erythrocytes from hemolysis. In 0.15M NaCl, the polyenes which foster rapid K^+ efflux (e.g., AmB) resulted in hemolysis; polyenes which cause slow K^+ efflus (e.g., AmBME) did not. Since hemolysis did not occur when sucrose was added at a concentration of 27mM, the polyene-induced hemolysis is almost certainly secondary to rapid osmotic changes rather than to a primary effect of polyene action. We have presented data previously suggesting that in C. albicans polyene-induced leakage of small molecules may not be solely responsible for fungicidal action (10). Regardless, K^+ leakage seems a good measure of polyene-induced membrane damage.

The toxicity of these polyenes toward other mammalian cells and toward liposome model membranes of known lipid composition is being investigated to try to define the nature of the polyene selective toxicity.

Acknowledgement

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