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Comparative *in vitro* studies on liposomal formulations of amphotericin B and its derivative, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME)★✪

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***N*-Methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME) is a semisynthetic derivative of the antifungal antibiotic amphotericin B (AMB). In contrast to the parent antibiotic, the derivative is characterised by low toxicity to mammalian cells and good solubility in water of its salts.**

Comparative studies on biological properties of free MFAME, AMB and their liposomal formulations were performed. To obtain liposomal forms, the antibiotics were incorporated into small unilamellar vesicles composed of dimyristoyl phosphatidylcholine (DMPC) and DMPC : cholesterol or ergosterol, 8:2 molar ratio. The effectivity of the liposomal and free forms of AMB and MFAME were compared by determination of fungistatic and fungicidal activity against *Candida albicans* ATCC 10261, potassium release from erythrocytes, and haemolysis.

The results obtained indicate that in contrast to AMB, incorporation of MFAME into liposomes did not further improve its selective toxicity.

Studies on the antagonistic effect of ergosterol and cholesterol on the antifungal activity of the antibiotics indicated that sterol interference was definitely less pronounced in the case of MFAME than in the case of AMB.

The lack of an effective and nontoxic drug for the treatment of a variety of systemic mycotic infections, especially common in patients with induced or acquired immunologi-

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Abbreviations: AMB, amphotericin B; DMPC, dimyristoyl phosphatidylcholine; Me_2SO, dimethyl sulfoxide; MFAME, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester; MFC, minimal fungicidal concentration; MIC, minimal inhibitory concentration; SUV, small unilamellar vesicles.

cal deficiencies, is the major problem in antifungal chemotherapy. The polyene macrolide antibiotic amphotericin B (AMB), which was introduced in the 1950s, still remains the most valuable drug against the widest spectrum of human mycotic infections with no major problems of resistance. However, application of AMB is hampered by its severe side effects, either acute or delayed and also by its insolubility in injectable aqueous media. To circumvent the latter disadvantage AMB is clinically administrated as buffered colloidal dispersion in sodium deoxycholate, Fungizon.

Many efforts have been made to develop less toxic formulations of AMB [1, 2]. The aim of optimising the pharmacological properties of AMB *via* chemical modification led, in our laboratory, to the synthesis of a series of water soluble AMB derivatives with preserved antifungal activity and diminished toxicity. Perhaps the most interesting derivative is *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME) (Fig. 1). This compound forms water soluble salts, retains the broad antifungal spectrum and potency of the parent antibiotic, and, in comparison with AMB, exhibits a two orders of magnitude lower toxicity towards mammalian cells *in vitro* and *in vivo*[1] [3–4]. The molecular basis of the selective toxicity improvement remains to be solved and is under investigation.

Another approach to improve AMB therapy was to use suitable carrier systems. Recent studies indicate, that AMB incorporation into lipidic structures, such as liposomes, significantly reduce the systemic toxicity of the antibiotic. Out of the numerous lipid formulations studied, three preparations are now either marketed for clinical application or undergoing preclinical studies. However, the goal was only partially achieved by the development of the lipid-based delivery system, because these new formulations are still considerably toxic and very expensive [2, 5].

The purpose of the present study was to compare the effect of liposomal delivery on the selectivity of the low toxic, water soluble MFAME and the highly toxic, water insoluble parent AMB.

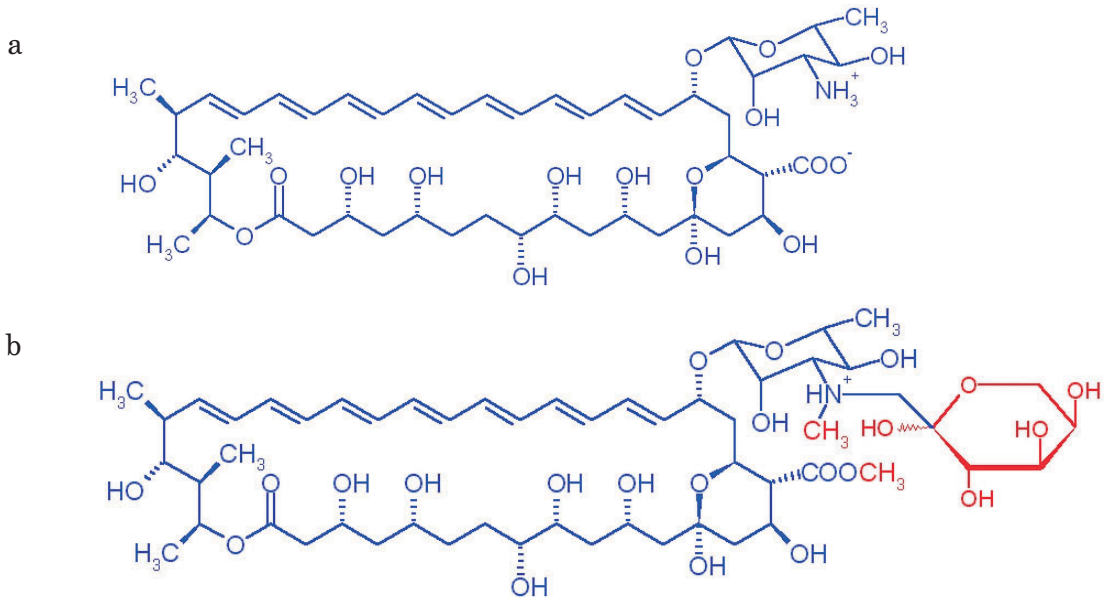

Figure 1. Structure of amphotericin B (AMB) (a) and *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME) (b).

[1]M.M. Bontemps-Gracz *et al.,* 7th *International Symposium on Molecular Aspect of Chemotherapy,* September 1999, Gdańsk, Poland, Abstracts p. 159.

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To obtain the liposomal form, antibiotics were incorporated into small unilamellar vesicles composed of dimyristoyl phosphatidylcholine (DMPC) and DMPC : cholesterol or ergosterol, at a 8:2 molar ratio. The effectivity of the liposomal and the free form of MFAME and AMB were studied *in vitro* by determination of the fungistatic and fungicidal activity against *Candida albicans* ATCC 10261, haemolysis and the extent of potassium release from erythrocytes.

Antagonistic effects of free ergosterol and cholesterol on the activity of MFAME and AMB against *C. albicans* ATCC 10261 were also tested, by determination of minimal inhibitory concentrationand (MIC), minimal fungicidal concentration (MFC), and potassium efflux. Very early studies on the mechanism of action of AMB and some other polyenes proved that sterols may prevent antifungal action of these antibiotics. This phenomenon was ascribed to a physicochemical interaction which occurs between polyenes and sterols in aqueous media. This interaction reduces the effective concentration of the antibiotic and increases the amount which must be added to inhibit growth of fungal cells [6]. In this manner the antagonistic effect of sterols reflects the antibiotic affinity to these sterols, which is often considered as one of the factors determing selectivity of AMB and its derivative [2].

MATERIALS AND METHODS

Chemicals. Polyene antibiotics: AMB was from Sigma, MFAME and its L-aspartate salt were synthesised in our laboratory according to the previously described method [3]. In all determinations, water soluble MFAME-L-aspartate was used, which in the text was abbreviated as MFAME. The purity of the substances was determined spectrophotometrically (ε = 160 000 M^{-1} cm^{-1} at 408 nm for methanolic solutions of AMB and its derivative). All concentrations given in the text are calculated for 100% pure compounds. Lipids: dimyristoyl phosphatidylcholine (DMPC), cholesterol and ergosterol were purchased from Sigma.

Cells and media. Candida albicans ATCC 10261 was grown at 30°C in a medium containing 1% bacto-pepton (Difco), 2% glucose. Human blood, citrate anticoagulated, was stored at 4°C for no more than two weeks. Just before use, erythrocytes were separated from plasma and leukocytes by centrifugation (10 min, 1 000 × ***g***, 4°C) and were washed three times with 155 mM sodium chloride (saline).

Preparation of liposomal AMB and MFAME. Small unilamellar vesicles (SUV) were prepared according to the procedure of Newman and Hung [7] from DMPC or DMPC and cholesterol or ergosterol mixed in a molar ratio of 8:2. Briefly, phospholipid and sterol were dissolved in chloroform. After evaporation of the solvent, the lipid film was dispersed in saline. To clarify the suspension, samples were sonicated above the transition temperature of the phospholipid for 15 min. Final lipid concentration in the suspension was 10^{-3} M. AMB or MFAME were incorporated into the liposomes in two ways: 1) solutions of antibiotics in Me_2SO were added to the SUV suspension, or 2) solutions of antibiotics in methanol were added to the lipids dissolved in chloroform and than, after solvent evaporation, SUV were prepared from the lipid-antibiotic mixture.

In all experiments SUV which did not contain antibiotics were used as a control. Before use, free or antibiotic containing vesicle suspensions were incubated at room temperature in darkness for 24 h. The total antibiotic concentration was determined spectrophotometrically according to the absorbance at 408 nm after dilution of 0.1 ml of the sample with 0.5 ml of methanol and 0.4 ml of chloroform.

Determination of MIC and MFC of free and liposomal AMB and MFAME. Serial two-fold drug dilutions were prepared in Me_2SO. Ten μl of these antibiotic solutions were added to 1 ml of liposome suspensions

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and incubated for 24 h at room temperature in the darkness. One-hundred μl of the liposomal formulations of the antibiotics was added to 1 ml of cell suspension in growth medium containing 10^5 cells/ml. In all samples final lipid concentration was 10^{-4} M. For MIC determination of free antibiotics, 10 μl of drug dilutions prepared in Me_2SO was added to 1 ml of saline. One-hundred μl of antibiotic solutions in saline was added to 1 ml of cell suspension containing 10^5 cells/ml. The lowest concentration of the antibiotic yielding no growth in liquid medium after 24 h of incubation at 30°C was defined as MIC. For MFC determination, a 10 μl aliquot taken from the sample in which cell growth was inhibited was plated on solid medium composed of 1% bacto-peptone (Difco), 2% glucose and 2% agar (Difco). The lowest antibiotic concentration in which no cell colonies were observed after 48 h of incubation at 30°C was defined as MFC.

Determination of permeabilizing and haemolytic activity of free and liposomal AMB and MFAME. A potassium selective electrode (F2002, Radiometer, Copenhagen) was placed in the liposome suspension tested. When the recorder reading stabilised, packed suspension of erythrocytes was added to obtain final erythrocyte concentration of 10^8 cell/ml and potassium level in the medium was monitored. The amount of potassium released was determined according to a calibration curve and expressed as the percentage of that obtained in a water haemolysed sample. At the same time the amount of haemoglobin released was determined. For this purpose, 0.5 ml of the sample was centrifuged and absorbance of the supernatant, diluted ten-fold with water, was measured at 540 nm with a colorimeter. The values for one hundred percent of haemolysis were obtained in a sample haemolysed in water. The same experiments were performed in the case of free AMB and MFAME. Antibiotics were dissolved in Me_2SO and diluted one-hundred-fold with saline. Then, packed erythrocytes were added, and potassium released into the medium was determined.

Determination of the activity of AMB and MFAME toward fungal cells in the presence of sterol. Sterols were dissolved in the mixture Me_2SO:methanol (1:1, v/v) at a concentration of 6.25×10^{-4} M. The antibiotics AMB and MFAME were dissolved in Me_2SO and serially diluted to concentrations one thousand-fold higher than final one in each sample. Four μl of antibiotic solution and 32 μl of sterol solution were mixed and incubated for 1 h at room temperature. For determination of the permeabilizing activity of the antibiotics, this mixure was filled up to 4 ml with cell suspension containing 10^8 cells/ml and the amount of potassium released was measured as described above. For MIC and MFC determination proper amounts of the antibiotic-sterol mixture were added to a cell suspension containing 10^5 cells/ml.

RESULTS

Liposomes and liposomal forms of MFAME and AMB

The ability of liposomes to reduce the toxicity of AMB against mammalian cells can be modulated by the characteristics of the lipid vesicles: the presence and type of sterol, phospholipid properties (electric charge, acyl chain length, presence of double bonds) and the lipid/antibiotic ratio. In these studies, DMPC small unilamellar vesicles were used. Such formulations of AMB were nontoxic to human erythrocytes and had good antifungal activity [8]. The lipid concentration of liposomes was held constant with changing antibiotic concentrations. In the initial phase of this study it was established that effectivity was the same regardless the antibiotic was introduced before or after liposome formation. Incorporation of the antibiotic into the lipid phase was indicated by a shift of absorption band characteristic for the monomeric form

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from 408 to 412 nm and disappearence of the band at 420 nm [8].

Antifungal activity

The MICs and MFCs of free AMB and MFAME, as well as their liposomal forms, against *Candida albicans* ATCC 10261 are presented in Table 1. The data indicate that the high fungistatic (MIC) and fungicidal (MFC) activity of AMB is preserved in MFAME. MIC and MFC of the liposomal formulations were dependent on the antibiotic and the vesicle lipid composition. AMB incorporated into sterol-free or cholesterol-containing DMPC-liposomes had the same fungistatic and fungicidal activity as free antibiotic, whereas that incorporated into ergosterol-containing liposomes was 5 times less active.

Incorporation of MFAME into liposomes diminished its fungistatic activity only in the case of ergosterol-containing liposomes. MFAME bound into sterol-free or cholesterol-containing liposomes had the same MIC as free antibiotic. In contrast, the fungicidal activity of the liposomal forms was independent of sterol presence and structure and was 10 times lower than the activity of free MFAME.

Protection by sterols

The effect of free ergosterol and cholesterol on the antifungal activity of AMB and MFAME was tested in growing cultures of *Candida albicans* ATCC 10261. Data in Table 2 indicate that the antagonistic effects were dependent on the sterol and the antibiotic. AMB was more sensitive to sterol interference than MFAME. For both antibiotics ergosterol was a more efficiently neutralising agent than cholesterol. In the presence of ergosterol MIC and MFC of AMB were increased 500- and 100-fold, respectively, whereas those of MFAME only 50- and 20-fold, respectively. Cholesterol increased MIC and MFC of AMB 10- and 20-fold, respectively and only 2-fold those of MFAME. The protective effect of sterols against membrane permeabilization was tested with resting cells of *Candida albicans,* suspended in saline. Potassium release from *Candida albicans,* induced by the antibiotics applied alone or in the presence of sterols (Table 2), indicated a similar antagonistic effect of ergosterol and cholesterol on the permeabilizing action of AMB and a definitely smaller influence of cholesterol than ergosterol on the effectivity of MFAME.

Table 1. Antifungal activity of free and liposomal AMB and MFAME.

MIC and MFC for *Candida albicans* ATCC 10261 were determined by serial dilution. Cells were incubated with the antibiotics for 24 h at 30°C.

Antibiotic	Liposome composition	MIC [μM]	MFC [μM]
AMB	free	0.1	0.5
	DMPC	0.1	0.5
	DMPC:ergosterol, 8:2	0.5	2.5
	DMPC:cholesterol, 8:2	0.1	0.5
MFAME	free	0.2	0.5
	DMPC	0.2	5
	DMPC:ergosterol, 8:2	0.5	5
	DMPC:cholesterol, 8:2	0.2	5

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Table 2. The effect of sterols on the antifungal activity and permeabilizing effect of AMB and MFAME.

MIC and MFC for *Candida albicans* ATCC 10261 were determined by serial dilution. Cells were incubated with the antibiotics and sterols for 24 h at 30°C. The final sterol concentration was 5×10^{-5} M. The extent of potassium release from the cells was determined with a potassium selective electrode at room temperature. *Candida albicans* ATCC 10261 cells were suspended in saline at 10^8 cell/ml. Antibiotic concentrations were: 10^{-6} M AMB, 5×10^{-6} M MFAME. The final concentration of each sterol was 5×10^{-5} M.

Antibiotic/sterol	MIC [μM]	MFC [μM]	Potassium efflux [% of control]
AMB	0.1	0.5	90
AMB/cholesterol	1.0	10	17
AMB/ergosterol	50	50	15
MFAME	0.2	0.5	95
MFAME/cholesterol	0.5	1.0	80
MFAME/ergosterol	10	10	20

Toxicity to human erythrocytes

The effectivity of free and liposomal forms of AMB and MFAME on human erythrocytes determined by the extent of potassium release is shown in Fig. 2. Free AMB at 10^{-6} M promoted complete potassium leakage (Fig. 2a). Both the kinetics and the final extent of potassium release indicated that at the same experimental conditions free MFAME was 100-fold less potent than the parent antibiotic (Fig. 2b). Liposomal formulations of AMB and MFAME were less effective than free antibiotics (Fig. 2 and Table 3). The lipid composition of liposomes was important only in the case of AMB. The most significant reduction of the permeabilizing activity, by about 90%, was observed for sterol-free vesicles. The efficiency of sterol-containing liposomal forms of AMB was dependent on the antibiotic/lipid ratio. In

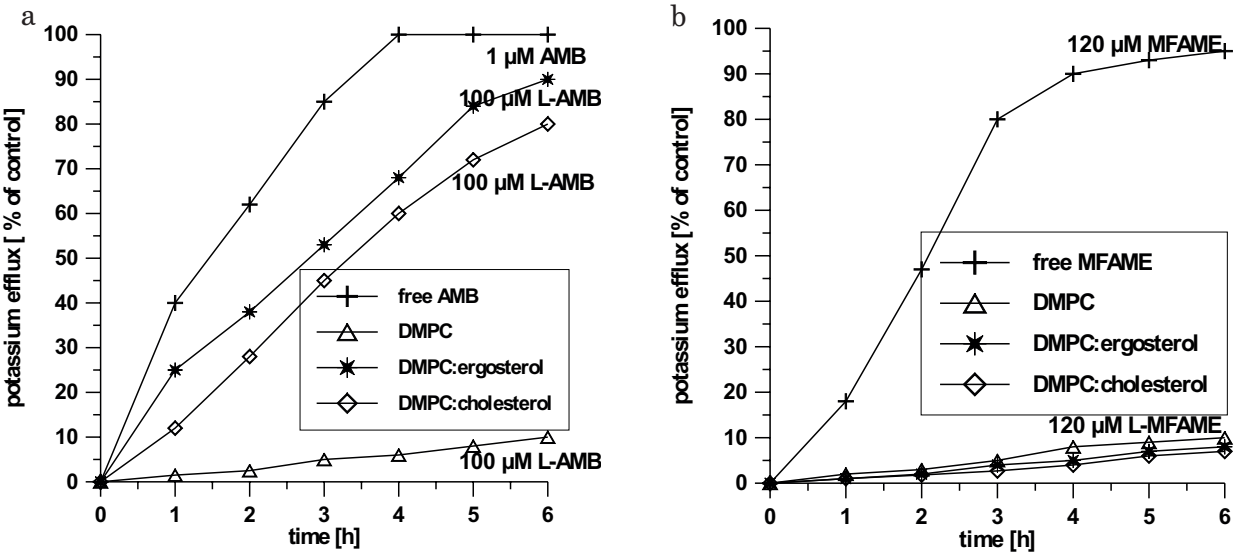

Figure 2. Time course of potassium release from human erythrocytes caused by free and liposomal AMB (a) and MFAME (b).

Human erythrocytes were suspended in saline at 10^8 cells/ml. The determination was performed at room temperature. The level of potassium in the cell suspension was measured with a potassium selective electrode.

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the case of MFAME all liposomal formulations exhibited similar, low permeabilizing activity.

Under the conditions studied, haemolysis was observed only for free AMB, and its extent was concentration- and time-dependent. For example, at 10^{-5} M complete hemolysis was observed after one hour of incubation of erythrocytes with the antibiotic. None of the liposomal formulations of AMB tested induced haemolysis. In the concentration range studied, neither free nor the liposomal forms of MFAME were haemolytic.

Table 3. Permeabilizing effect of free and liposomal AMB and MFAME on human erythrocytes.

The concentration of human erythrocytes was 10^8 cells/ml. The determination was performed at room temperature.

Antibiotic	Liposome composition	Potassium efflux after 6 h of incubation [% of control]
1 μM AMB	free	100
20 μM AMB	DMPC	5
	DMPC:ergosterol, 8:2	8
	DMPC:cholesterol, 8:2	7
50 μM AMB	DMPC	8
	DMPC:ergosterol, 8:2	20
	DMPC:cholesterol, 8:2	22
100 μM AMB	DMPC	10
	DMPC:ergosterol, 8:2	90
	DMPC:cholesterol, 8:2	80
120 μM MFAME	free	95
120 μM MFAME	DMPC	10
	DMPC:ergosterol, 8:2	8
	DMPC:cholesterol, 8:2	7

DISCUSSION

An new approach of reducing the toxicity of amphotericin B, but maintaining its antifungal activity, would represent a significant clinical advance in the management of fungal infections.

Superiority of liposome-encapsulated AMB in *in vitro* and *in vivo* studies has been clearly demonstrated. Many liposomal AMB formulations displayed reduced toxicity to mammalian cells (erythrocytes, macrophages, renal tubular cells), while maintaining activity against yeast. Ambisome, liposomal AMB which is now available as a drug is still considerably toxic, and the extremely high price practically excludes it from general use [2].

Our recent results have demonstrated that essential diminishment of AMB toxicity can be achieved by proper chemical modification of the parent antibiotic. Perhaps the most interesting derivative obtained was *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME) exhibiting radically improved selective toxicity [3–4]. The experiments presented in this report were designed to gain a better understanding of the molecular basis of the selective toxicity of this compound by comparing the properties of the toxic AMB and

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the nontoxic MFAME bound with liposomes, as well as by determining antagonistic effects of ergosterol and cholesterol on the antifungal activity of these drugs.

It is generally accepted that the toxic effect of AMB is a consequence of increased cell membrane permeability due to formation of specific permeabilizing species (channels) with participation of the membrane located sterols. The formation of a channel by AMB needs a higher threshold of concentration for cholesterol-containing, than ergosterol-containing membrane. A relation between the aggregation state of AMB in solution and its differential interaction with cholesterol- and ergosterol-containing membranes has been shown [9–12]. Liposomes seem to sequester the drug and can be considered as AMB reservoires, from which the drug is progressively released. Low concentration of free AMB resulted in an antifungal effect without toxicity against cholesterol-containing cells.

MFAME is different from AMB with respect to the hydrophilic-lipophilic balance, charge, and solubility in aqueous media. The existence of a link between the aggregation state of MFAME and its effect on human erythrocytes has been demonstrated [13]. The behaviour of the liposomal formulations of the positively charged and water-soluble MFAME could be different than of those of the parent AMB, which is zwitterionic and insoluble in water.

The results obtained show that MFAME incorporated into small lipid vesicles retains low toxicity towards human erythrocytes. Only ergosterol incorporated into the vesicles diminished the fungistatic action of both antibiotic and the fungicidal action of AMB. At the same time, the fungicidal effect of MFAME was reduced 10-fold independently of the lipidic composition of the liposomes.

In summary, we have shown that due to the decreased fungicidal effect the liposomal formulations of MFAME tested are not advantageous. It should be stressed, however, that under the same experimental conditions free MFAME was 100-fold less toxic towards human erythrocytes than AMB and its toxicity was at the level of liposomal AMB.

AMB was more sensitive to the antagonistic effect of free sterols than MFAME and for both antibiotics the protective effect of ergosterol was stronger than of cholesterol. This indicates that both antibiotics exhibit an ability to differentiate between ergosterol and cholesterol but in the case of MFAME this ability is better pronounced. However, the present results concerning the antibiotics tendency to interact with ergosterol and cholesterol, as well as previously published data [14], show, that the distinctly diminished toxicity, together with maintained good antifungal activity of MFAME can not be explained just by differences between the antibiotic's affinities for ergosterol and cholesterol. Our latest studies on MFAME and AMB interaction with bacterial (*E. coli*), fungal (*C. albicans*) and mammalian (K 562) cells, measured by the energy transfer method, indicate that the presence and structure of sterols in the membrane do not determine the antibiotic's ability to bind with a cell[2]. Thus, the differences between the selective toxicities of AMB and its derivative could be expressed at the supramolecular level of the membrane permeability pathway formation.

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