Acta Biochimica Polonica

Vol. 47 No. 1/2000

121–131

QUARTERLY

N-Methyl-*N*-D-fructosyl amphotericin B methyl ester (MF-AME), a novel antifungal agent of low toxicity: Monomer/micelle control over selective toxicity✪★

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Received: 25 October, 1999

Key words: amphotericin B, reactional drug design, selective toxicity

Rational chemical modification of amphotericin B (AMB) led to the synthesis of sterically hindered AMB derivatives. The selected optimal compound, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester (MF-AME) retains the broad spectrum of antifungal activity of the parent antibiotic, and exhibits a two orders of magnitude lower toxicity *in vivo* and *in vitro* against mammalian cells. Comparative studies of MF-AME and AMB comprising the determination of the spectroscopic properties of monomeric and self-associated forms of the antibiotics, the investigation of the influence of self-association on toxicity to human red blood cells, and of the antibiotic-sterol interaction were performed.

On the basis of the results obtained it can be assumed that the improvement of the selective toxicity of MF-AME could in part be a consequence of the diminished concentration of water soluble oligomers in aqueous medium, and the better ability to differentiate between cholesterol and ergosterol.

★Presented at the 7th International Symposium on Molecular Aspects of Chemotherapy, September 8–11, 1999, Gdańsk, Poland.

✪This work was supported in part by grant 4 P05F 035 16 from the State Committee for Scientific Research (KBN, Poland) and by Chemical Faculty of Technical University of Gdańsk.

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Abbreviations: AMB, amphotericin B; CD, circular dichroism spectroscopy; c.f.u., colony forming unit; EH_{50}, compound concentration which causes 50% of haemoglobin loss from erythrocytes; $EK_{50,}$ compound concentration which causes 50% of potassium loss from erythrocytes; MF-AME, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester; MF-AME L-aps, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester L-aspartate; MIC, minimal inhibitory concentration; PBS, phosphate buffered saline; UV-VIS, ultraviolet, visible light spectroscopy.

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 immunological deficiencies, is the major problem in antifungal chemotherapy. The polyene macrolide antibiotic amphotericin B (AMB) is the most effective, currently available, drug for the treatment of a wide variety of deep-seated mycotic infections in humans. However, clinical application of AMB is limited by a number of severe short or long term side effects.

The mechanisms of the antifungal activity of AMB as well as of its toxicity for the host are not yet completely clear. It is generally assumed that the fundamental reason for the insufficient antifungal selectivity of the drug is its ability to perturb both fungal and mammalian cellular membranes by interaction with endogenous sterols. Cell sensitivity to AMB is determined by sterol structure. The selective toxicity of AMB for fungi is the result of its capacity to bind more strongly to ergosterol present in the fungal cell membrane than to cholesterol in mammalian ones [1].

AMB is virtually insoluble in water. In aqueous media it undergoes self-association and is present as a mixture of monomers and various soluble and insoluble aggregates in equilibrium. Recently it has been shown that the state of the antibiotic in solution is another essential factor influencing its selective toxicity. It has been found that AMB in monomeric form is significantly less toxic toward mammalian cells than toward fungal cells, whereas AMB in the form of water soluble aggregates is nonselective and toxic for both types of cells [2].

Thus both the antibiotic sterol affinity and the state of the antibiotic in aqueous medium are important factors which should be taken into account in the rational design of new AMB derivatives with improved pharmacological properties. Both features can be influenced by chemical modification of the AMB molecule on its polar head [2–6].

The results of our theoretical and experimental studies comprising the recognition of the molecular nature of the antibiotic–sterol primary complex and its contribution to biological properties of active compounds as well as the identification of the structural factors determining solubility of AMB and its derivatives in aqueous media led to the synthesis of a series of sterically hindered derivatives. The compounds are substituted Amadori rearrangement products obtained with various sugars and amphotericin B. The selected optimal compound *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester L-aspartate (MF-AME L-asp) [7] is perfectly water soluble, retains the broad spectrum antifungal activity of the parent antibiotic, and exhibits a high activity against multidrug resistant fungal cells. The compound exhibits a two orders of magnitude lower toxicity against animal cells in *in vitro* experiments and its LD_{50} in mice is 400 mg/kg, compared to 6 mg/kg for AMB [7].

The purpose of the present work is to explain to what extent the state of MF-AME in solution and its differential affinity to ergosterol and cholesterol may explain such a substantial improvement of its selective toxicity.

MATERIALS

Amphotericin B (AMB) was a generous gift from Bristol-Meyers Squibb. *N*-Methyl-*N*-D-fructosyl amphotericin B methyl ester (MF-AME) and its water soluble salt with L-aspartic acid (MF-AME L-asp) were synthesised in our laboratory [7].

Amphotericin B, MF-AME and MF-AME L-asp solutions were prepared daily at 1 mg/ml or 10 mg/ml in *N,N*-dimethyl formamide (AMB and MF-AME) or water (MF-AME-L-asp), respectively.

Cholesterol and ergosterol were purified by repeated crystallisation from ethanol. The sterols stock solutions in chloroform were prepared daily at 10 mg/ml.

Human erythrocytes, from a local blood bank, were chosen as model cholesterol containing cells. As model cells for testing the antifungal activity *Candiada albicans* ATCC 10261 was chosen.

Isotonic, phosphate buffered saline (PBS) was of 155 mM NaCl and 7 mM Na_2HPO_4, pH 7.4. The buffer stability was controlled every two weeks.

METHODS

In vitro *susceptibility testing*. Antifungal activity was determined on *Candida albicans* ATCC 10261 by the serial dilution method on the BG liquid medium containing Bacto-Peptone (Difco), 0.5% and glucose, 1%. The medium was inoculated with tested microorganism (final concentration 0.5×10^3c.f.u./ml) and incubated for 24 h at 30°C. The minimal inhibitory concentration (MIC) was defined from dose response curves as the lowest compound concentration that completely inhibited fungal visible growth.

Erythrocytes preparation. Human blood was stored at 4°C for no more than three weeks. Before the experiment human erythrocytes were separated from plasma and leukocytes by centrifugation at 800 × ***g*** for 15 min at 4°C. Then erythrocytes were washed three times with PBS.

Potassium release from human erythrocytes — dose response. Appropriate concentrations of antibiotics were incubated for 60 min at 37°C and then each sample was divided into halves. One part of each sample was centrifuged at 4000 × ***g*** for 20 min and the supernatant was collected. Human erythrocytes were added, in a volume which gave final cell number 2×10^7cells/ml, to supernatants and non centrifuged antibiotic suspensions. Samples were incubated for 60 min at 37°C, with shaking. Than they were centrifuged 2000 × ***g*** for 15 min and the amount of potassium released was measured with flame photometry in the supernatant of each sample. The amount of potassium in the supernatant was expressed as the percentage of total potassium measured in the sample obtained by total hemolysis of the erythrocytes.

Kinetics of potassium release. The packed cells suspension (200 μl) was added to 5 ml of PBS. Then a potassium selective electrode (F2002 Radiometer Copenhagen) was introduced to cell suspension. When the recorder reading stabilised, antibiotic solution was injected and the potassium level in medium was monitored continuously for 40 min. One hundred percent of potassium release corresponded to potassium level in the hemolysed sample.

Self-association studies. Appropriate amounts of antibiotics were added to PBS and incubated for 60 min at 37°C, with shaking. UV-VIS spectra and circular dichroism (CD) spectra were recorded at the range of 500–300 nm. The determination of the ratio of different species was done according to the molar absorption coefficient at 408 nm. Insoluble aggregates: the precipitate separated by centrifugation at 4000 × ***g*** for 20 min was suspended in the original volume of PBS and diluted (1 : 1) with methanol.

Monomers: concentration of monomers was determined in supernatant of centrifuged samples.

Soluble oligomers: the amount of soluble oligomers was taken as the difference between the total concentration of antibiotic and the concentration of monomers and insoluble aggregates.

Antibiotic–sterol complexation. Required amounts of antibiotic and sterol stock solutions were diluted with ethanol and then mixed with 1/4 volume of PBS. The mixture was incubated for 60 min at 37°C and absorbance was measured at 408 nm. The percentage of antibiotic bound to sterol (X%) was calculated according to the equation:

$$X\% = [(C_a - C_{aw}) \times 100\%]/C_a$$

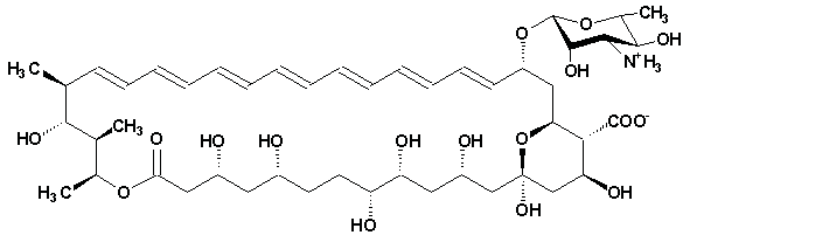
where the C_{aw} — concentration of free antibiotic in solution in the presence of sterol, C_a — total antibiotic concentration (2×10^{-6} M.).

Different sterol : antibiotic molar ratios (R) were obtained by mixing different sterol amounts with a constant antibiotic amount.

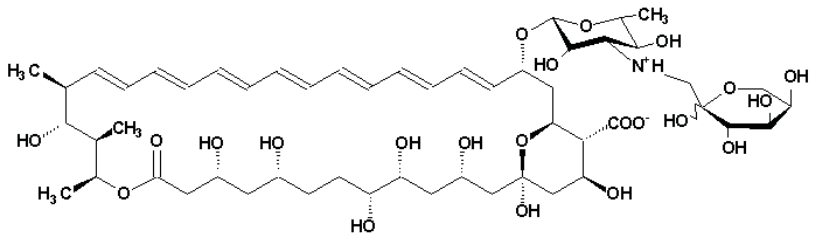
RESULTS AND DISCUSSION

Compounds structures and solubility

Molecular structures of AMB and its derivative used in this study – MF-AME – are presented in Fig. 1. AMB is an amphipathic molecule with a hydrophobic part consisting of seven conjugated double bonds and several hydrophilic substituents localised at the macrolide ring as well as in the "polar head" containing a carboxyl group at C-16 and an aminosugar at C-19. It is also amphoteric due to the presence of carboxyl and amino groups, both of which are charged at neutral pH. AMB, due to its amphipathic and amphoteric properties, is poorly soluble in aqueous media. Monomolecular AMB solution in aqueous media was obtained only at the compound concentration lower than 10^{-7}M. Above this concentration monomers of AMB undergoe self-association and form water soluble oligomers and water insoluble aggregates. The tendency of AMB to self-association can be rationally controlled by chemical modification. It has been evidenced that a net electric charge in the antibiotic molecule prevents self-association [3]. The majority of water soluble derivatives of AMB: salts of esters, trimethylammonium derivatives of methyl ester, amides, esters of *N*-aminoacyl derivatives, possess a net charge in the polar head of the molecule. Zwitterionic derivatives *N*-aminoacyl and *N*-fructosyl are more soluble in aqueous media only after the formation of salts. AMB does not form salts between pH 4 and 9, probably due to the presence of a strong intramolecular electrostatic interaction between charges of opposite signs. A net charge on the molecule increases solubility due to the repulsion between charges of the same sign. The concentration of AMB monomers at pH 2 and 10, in which the molecule is positively or negatively charged, respectively, was several times higher than at pH 7.4 [3]. Also the introduction of proper hydrophilic substituents into the polar head of the macrolide (AMB–oligoethylene glycol conjugates) led to a significant increase in the critical micelle concentration of the antibiotic [6]. MF-AME is of basic character and, with organic and inorganic acids [7], forms salts, which are water soluble at neutral pH. In



Amphotericin B



N-methyl-N-D-fructosyl amphotericin B methyl ester

Figure 1. Structures of compounds tested.

MF-AME the amphipathic character of the parent antibiotic is preserved, but esterification of the carboxyl group confers a positive charge onto the molecule. The introduction of a bulky hydrophilic substituent in the amino group changes hydrophilic/lipophilic balance of the molecule.

Biological properties

Biological activities of MF-AME and AMB were compared against *Candida albicans* ATCC 10261 and human erythrocytes considered as examples of pathogenic and host cells, respectively. The antifungal activity was expressed as MIC and the mammalian cell toxicity was expressed as EK_{50} and EH_{50}. Data presented in Table 1 indicate that for MF-AME the antifungal activity is well preserved, whereas the toxicity to animal cells is remarkably reduced. In contrast to AMB, the derivative is practically not haemolytic. Even after 24 h incubation of erythrocytes with MF-AME at a concentration $>10^{-4}$M in PBS no haemolysis was observed. In the case of potassium release the effectivity of MF-AME was almost 300 fold lower than that of AMB.

Kinetics of potassium efflux

The kinetics of potassium efflux from erythrocytes (Fig. 2) indicate a very low permeabilizing efficiency of the derivative. For AMB at 5×10^{-6} M 10 min was sufficient to cause complete potassium release. For MF-AME even at a concentration 10 times higher (5×10^{-5} M) the release of potassium was slow and only partial even after prolonged incubation time. When AMB was added to cell suspension sometime after MF-AME the response of red cells was much slower than in the case of AMB alone. This suggested that both compounds interact with the same target. However, even at a concentration 10 times lower, AMB was able to compete with MF-AME for the active site and to increase potassium leak from red cells.

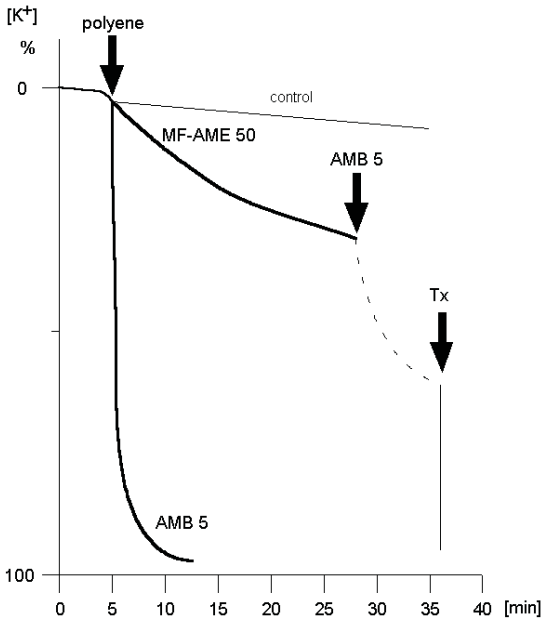


Figure 2. Kinetics of potassium release from eythrocytes.

AMB and MF-AME were added to cells suspension at 5 μg/ml and 50 μg/ml, respectively. Erythrocytes were suspended in PBS at 2×10^8 cells/ml. Temp. 22°C, Tx, Triton X-100.

Table 1. Biological properties of MF-AME and AMB

Compound	MIC [μg/ml]	EH50 [μg/ml]	EK50 [μg/ml]
AMB	0.2	1.7	0.7
MF-AME	0.8	>300	195

EK_{50} – compound concentration which causes 50% of potassium loss from erythrocytes.

EH_{50} – compound concentration which causes 50% of haemoglobin loss from erythrocytes.

MIC – minimal compound concentration which completely inhibits fungal growth. MIC was determined on *Candida albicans* 10261.

Spectroscopic properties

The spectroscopic properties of AMB are determined by the presence of the all-*trans* heptaenic chromophore. UV-VIS spectra of AMB and MF-AME in the range of 300–420 nm are characterised by four bands, whose intensities and positions are solvent dependent. UV-VIS spectra of AMB and MF-AME ethanolic solutions are identical, with four bands of decreasing intensity at 408, 385, 365, and 348 nm (Fig. 3). Monomeric forms of AMB and MF-AME show identical band intensities and proportions are the same. Molar absorption coefficients for both compounds are the same and equal to $\varepsilon = 160\,000\ M^{-1}\,cm^{-1}$. Substitutions at the polar head of AMB had no effect on properties of the polyenic chromophore, therefore, spectroscopic methods which have been successfully used in the studies of AMB self-association and AMB–sterol interaction can be also applied in the case of MF-AME.

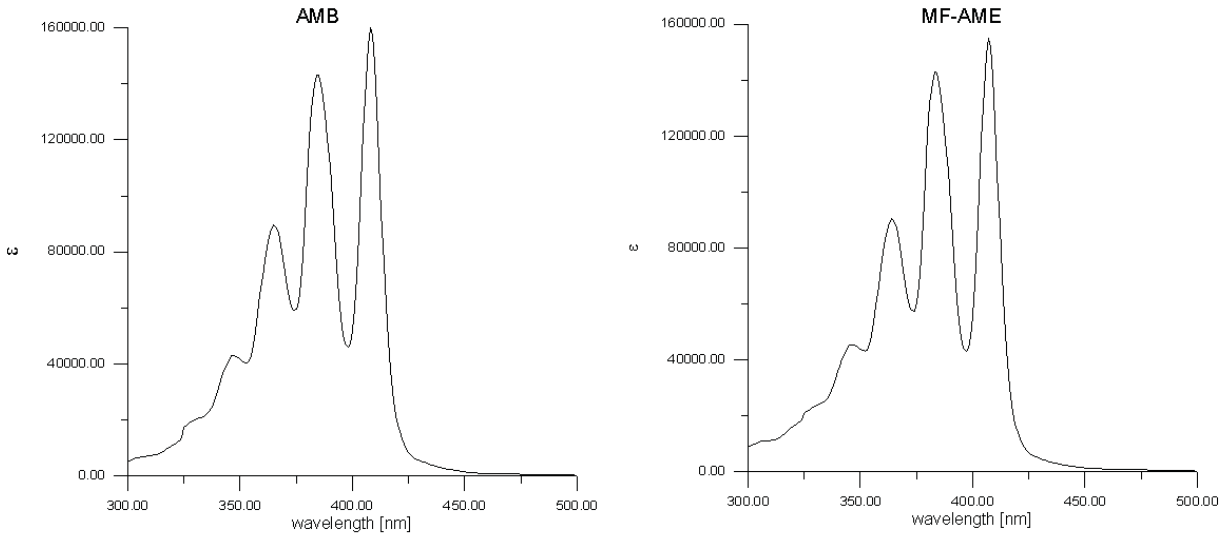


Figure 3. UV-VIS spectra of AMB and MF-AME in ethanol.

Self-association

In aqueous media three forms of AMB coexist: the monomer, water soluble oligomers and water insoluble aggregates. Proportions of particular species are dependent on many factors. The most important ones are: medium composition and pH, organic solvent and antibiotic concentration in stock solution and temperature. In previous studies it has been shown that the induction of permeability to K^+ in erythrocytes required some threshold AMB concentration above which antibiotic self-association begins. It has been shown that the activity of AMB to cholesterol containing membranes is restricted to water soluble self-associated AMB oligomers, whereas monomeric AMB and water insoluble aggregates were inactive [2, 8]. The behaviour of MF-AME in aqueous solution has not been studied before. MF-AME, especially as the L-aspartate salt, is a compound of high water solubility. It means that up to a comparatively high concentration it exists in forms which do not precipitate from pure water or other aqueous media like isotonic glucose. However, precipitation was observed in the presence of salts. Experiments on erythrocytes and other animal cells are usually performed in media containing relatively high salts concentration, like saline or PBS. Self-association of MF-AME and AMB in PBS were compared,

following changes of the spectroscopic properties of the polyenic chromophore by UV-VIS and CD spectroscopy.

UV-VIS spectra

In Fig. 4 the absorption spectra of MF-AME and AMB in PBS are presented. For MF-AME as well as for AMB the spectra change gradually with increasing antibiotic concentration. Antibiotic self-association is indicated by a decrease of the 408 nm band intensity and simultaneous appearance of a wide intense band around 340 nm. For AMB, absorption at 408 nm has been observed only for the monomeric form, and the intensity of this band was used also for the determination of MF-AME monomer concentration. In general, spectra characteristic for the associated forms of AMB and MF-AME are very similar

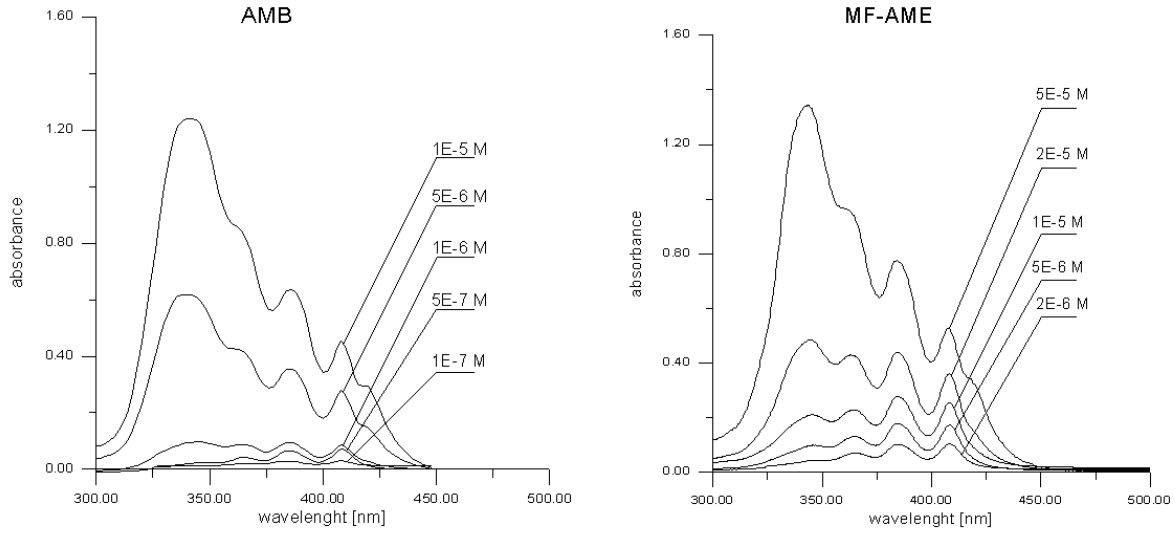


Figure 4. Self-association of compounds in PBS. UV-VIS spectra.

Appropriate amounts of compounds were added to PBS buffer and incubated for 60 min at 37°C, with gentle shaking.

although the band characteristic for self-associated species appeared for MF-AME at a higher concentration and for a given concentration had lower intensity than in the case of AMB.

Thanks to the characteristic changes in the UV-VIS spectrum connected with compound self-association we could calculate the concentrations of different forms of MF-AME in PBS. When the total concentration of the compound increased the concentration of insoluble aggregates increased parallelly. The concentration of soluble species (monomers and water soluble oligomers) reached saturation when the total antibiotic concentration was 3×10^{-5} M (Fig. 5a). Above this concentration the total amount of MF-AME monomers is comparable to that of AMB. AMB soluble aggregates concentration is almost three times higher than that of MF-AME (Fig. 5b).

CD spectra

CD spectroscopy appeared to be especially useful in the studies of AMB self-association. It is due to the fact that the spectra of the monomeric and self-associated forms are very different. The spectra of AMB and MF-AME are compared in Fig. 6. The spectra of the monomolecular solution of AMB and MF-AME are both characterised by four bands of very low intensity at the same wavelength as in the absorption spectra. The CD spectrum of the monomer is about 100 times

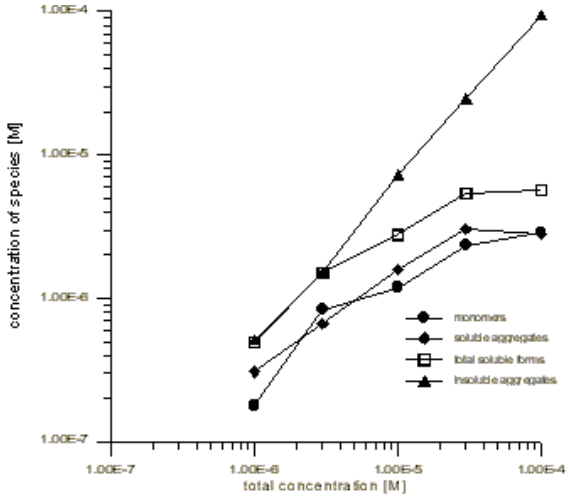


Figure 5a. Concentrations of different antibiotic forms in PBS.

Antibiotic was dissolved in dimethylformamide, diluted to appropriate concentration with PBS and incubated for 60 min at 37°C. Proportions of antibiotic monomers and aggregated species were determined by absorbance in centrifuged and non centrifuged samples.

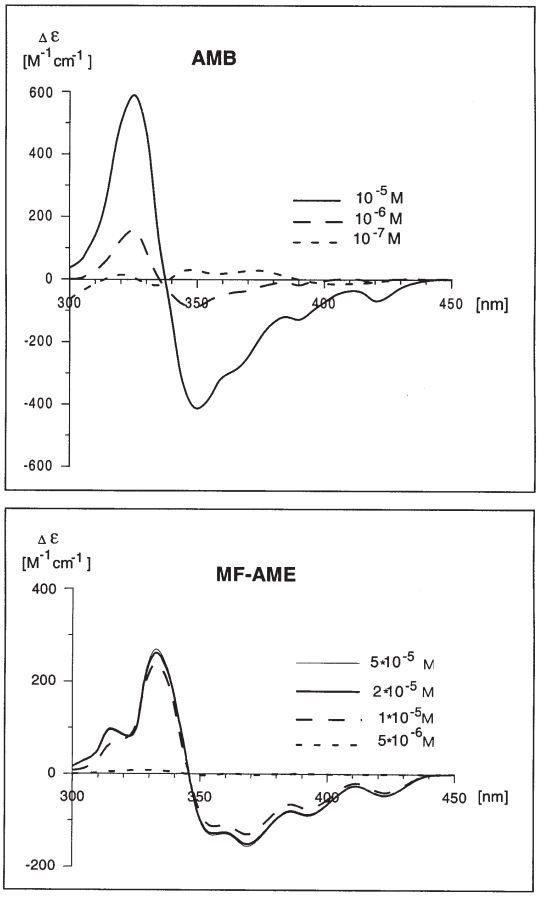


Figure 6. Self-association of compounds in PBS. CD spectra.

Appropriate amounts of compounds were added to PBS buffer and incubated for 60 min at 37°C, with gentle shaking.

weaker than of the associated form. Self-association of AMB is indicated by the appearance of the strong dichroic doublet centred at 340 nm (positive peak at 325 and negative peak at 350 nm). For AMB the amplitude of this band reflected the concentration of soluble aggregates. Variation of other spectral characteristics, which are related to conformational changes of the aggregates, were not observed in these experiments. CD spectra of AME and *N*-acetyl AMB were the same as of AMB [2]. CD spectra of MF-AME were different from those of AMB in respect to shape, bands positions and intensities, and concentration dependence. In the case of MF-AME a new positive band at 320 nm was observed, the dichroic doublet was centered at 346 nm, appeared at a higher concentration and for the

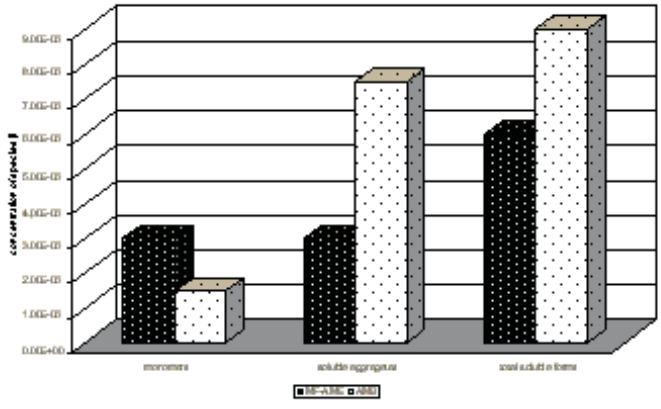


Figure 5b. Calculated concentrations of different antibiotic species at saturation.

concentration range 10^{-5} M to 5×10^{-5} M its intensity was constant. These results suggest that in the case of MF-AME, UV-VIS and CD spectra detect different self-associated species. The amount of those detected by CD is concentration independent in the range studied. The differences in spectrum shape and doublet position suggest that the structure of soluble oligomers formed by the derivative and parent antibiotic are not the same.

Permeabilizing activity *versus* state of the antibiotic in solution

We correlated our results on compounds self-association with their permeabilizing activity on human erythrocytes (Fig. 7). The results obtained confirmed the assumption that for MF-AME, like for AMB, water soluble oligomers are the form active toward mammalian cells. The concentration of MF-AME soluble aggregates in PBS is significantly lower than for AMB (Fig. 5a) and this fact can be considered as one of the factors responsible for the significant diminishment of MF-AME activity toward mammalian cells. In PBS, due to the salting out effect, formation of insoluble aggregates was observed at a relatively low concentration of MF-AME. Insoluble aggregates can be considered as a source of monomeric forms, inactive towards cholesterol containing membranes. The improvement of selective toxicity observed for heat induced AMB superaggregates and liposomal AMB has been explained in a similar way [9–11].

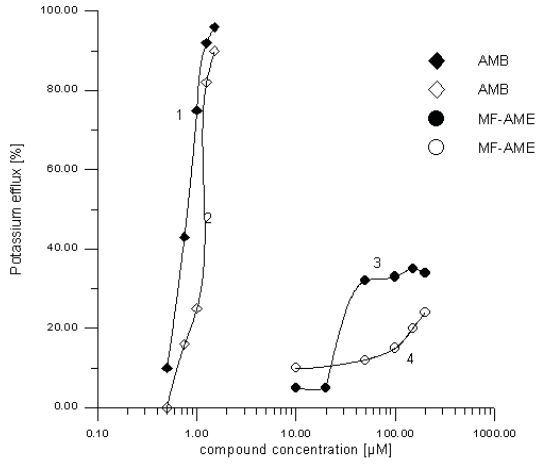


Figure 7. Potassium efflux–dose response curves.

Appropriate amounts of antibiotic, dissolved in dimethylformamide, were added to PBS and incubated for 30 min at 37°C. Human red blood cells, at final concentration of 2×10^7 cells/ml, were added to: 1, 3, non centrifuged sample; 2, 4, centrifuged sample. Cell suspensions were incubated for 60 min at 37°C. Concentration of potassium in supernatant was determined by flame photometry.

Antibiotic–sterol complexation

The aim of the experiment was to establish MF-AME affinity toward cholesterol and ergosterol molecules in aqueous media.

The estimation of polyene–sterol affinity was based on changes in the antibiotic UV-VIS spectra occurring upon interaction with the sterol. The percentage of sterol bound antibiotic was calculated as the percentage of absorption at 408 nm decrease compared to free antibiotic spectra. Experimental conditions in which sterol remained soluble and the polyenes were in monomeric forms were chosen [4].

The amount of sterol bound MF-AME was independent of the ergosterol: antibiotic ratio and increased with increasing cholesterol : antibiotic ratio. Both antibiotics bind to ergosterol more easily than to cholesterol (Fig. 8). In the case of MF-AME, the discrimination between ergosterol and cholesterol was more pronounced than for AMB.

In conclusion it can be said that the differences in water soluble oligomers concentration and affinity toward cholesterol and ergosterol between MF-AME and AMB can not fully explain the drastic diminution of MF-AME toxicity toward mammalian cells. It is generally accepted that biological activity of amphotericin B is a consequence of increased membrane permeability due to formation of a specific structure (permeabilizing unit) between the antibiotic and membrane located

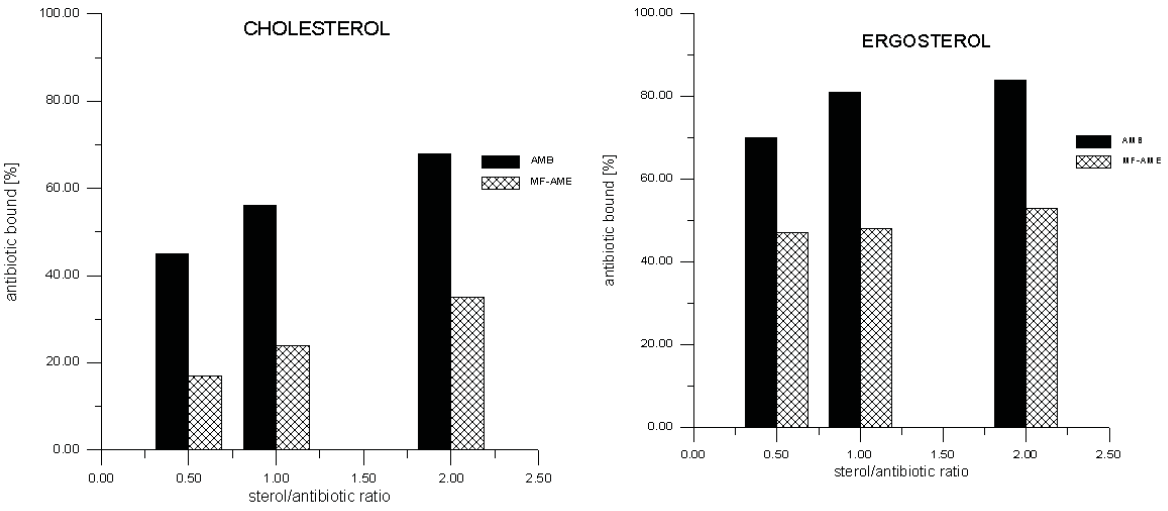


Figure 8. Antibiotic–sterol complexation.

Antibiotics were added to ethanol/PBS (1:4, v/v) solution at 2×10^{-6} M and incubated with sterols for 60 min at 37°C. The intensity decrease of the band at 408 nm was used to establish unbound antibiotic concentration in supernatants.

sterols. The structure of sterols determines formation, stability and permeabilizing profile of the unit. It has been shown that the modification of the "polar head" of the AMB changes the properties of the permeabilizing unit to different extent depending on sterol structure. The improvement of the selective toxicity of some AMB derivatives was explained on this basis [12–14].

There is no data on the properties of the permeabilizing unit formed by MF-AME and the two sterols. Differences in the properties of the units formed in ergosterol and cholesterol containing membranes have to be taken into consideration for the complete explanation of the selective toxicity improvement observed for MF-AME.

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