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MFAME, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester, a new amphotericin B derivative of low toxicity: relationship between self-association and effects on red blood cells

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Abstract

In aqueous solutions *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME), a novel amphotericin B derivative with low animal toxicity, similar to its parent antibiotic, exists in three forms: monomeric, soluble and insoluble aggregates in equilibrium [1]. The aim of our work was to examine the influence of medium composition on the MFAME self-association and the relationship between MFAME self-association and its toxicity towards red blood cells. The toxicity of MFAME in aggregated state towards red blood cells was tested by measuring the induction of potassium leakage and extent of haemolysis. The proportions of antibiotic species present in various aqueous media were determined by analysis of the UV-Vis spectra as a function of the antibiotic concentration. Numeric decomposition of the spectra allowed identification of four spectral species present in MFAME solutions: monomeric and three aggregated forms. Our results indicate that these aggregates, named type I, type II and type III, are different in terms of spectral properties, as well as effectiveness towards red blood cells. Soluble aggregate types I and III are the active forms of MFAME towards erythrocytes. The medium composition seems to be the main factor determining which type of antibiotic aggregate prevails in solution.

Keywords: Amphotericin B; Amphotericin B derivative; Rational drug design; Self association; Selective toxicity

1. Introduction

The polyene macrolide antibiotic amphotericin B (AMB) is widely used in clinics as a chemotherapeutic agent of choice for the treatment of systemic fungal infections. The main advantages of the drug are: broad antifungal spectrum, fungicidal activity and low frequency of occurrence of clinical strains with developed specific resistance towards this compound. However, clinical use is limited by very low selective toxicity of AMB. Moreover, poor water solubility also makes its application difficult. The undesirable properties of the drug stimulate the research on improvement of the pharmacological properties of the antibiotic. The theoretical and experimental studies on the mechanism of action of AMB and its derivatives, which were carried out in our laboratory, indicated that rational chemical modification of AMB molecule seems to be the most appropriate way to reach the goal. The strategy of designing the modified AMB of low toxicity and good water solubility, developed in our laboratory, led to the synthesis of a series of sterically hindered AMB derivatives [2,3]. The most interesting compound of this group is *N*-methyl-*N*-D-fructosyl AMB methyl ester (MFAME). This compound retains the broad antifungal spectrum and potency of parent antibiotic comprising MDR fungal strain [4]. At the same time it exhibits two orders of magnitude lower toxicity than AMB towards animal cells in in vitro [5] and in vivo experiments [6]. It is also of importance that MFAME is able to form water-soluble salts. The structures of AMB and MFAME are presented in Fig. 1.

AMB is a membrane active compound. However, it should be stressed that the mechanism of action of

Abbreviations: AMB, amphotericin B; CD, circular dichroism spectroscopy; DMSO, dimethyl sulphoxide; MFAME, *N*-methyl-*N*-D-fructosyl amphotericin B methyl ester; PBS, phosphate-buffered saline; RBC, red blood cells; UV-Vis, ultraviolet, visible light spectroscopy

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AMB is very complex and, in spite of intensive studies, not completely understood (reviewed in [7]). Effects induced in fungal as well as mammalian cells depend on many factors, such as the antibiotic concentration, medium composition and many other experimental conditions.

It is generally accepted that cell sensitivity to antibiotics is determined by the structure of membrane-located sterols: the ergosterol in fungal cells, and the cholesterol – the principal sterol of mammalian cells. The mechanism of action on both types of cells is of a similar nature: AMB interacts with membrane-located sterol, AMB–sterol complexes associate and form transmembrane channels through which free diffusion of many components essential for cell life occurs, leading to cell growth inhibition or cell death. The selective toxicity of AMB is based on the differences in the affinity of the drug to both sterols and differences in the properties of the permeability pathway formed in ergosterol- and cholesterol-containing membranes (reviewd in [7]).

AMB is practically insoluble in water. In aqueous media it undergoes self-association and is present as a mixture of monomer, soluble and insoluble aggregates in equilibrium. It was shown that the degree of self-association of AMB in aqueous media is also an important factor influencing its ergosterol/cholesterol selectivity [8–11]. The monomeric form is of much higher selectivity than soluble aggregates. It was postulated that AMB in monomeric form is not able to create permeability pathways in the cholesterol-containing cells and model membranes. In contrast, soluble aggregates of AMB are effective on cholesterol as well as ergosterol containing cells and membranes [8–11]. In our previous study it was shown that MFAME-L-aspartate exhibits the same mode of action on fungal cells as the parent antibiotic [4], and is dramatically less haemolytic and exhibits significantly lower permeabilizing activity towards red blood cells (RBC) than AMB [1,13]. It was suggested that the improvement of the selective toxicity of water soluble MFAME salts could in part be a consequence of diminished tendency to self-association in aqueous media, resulting in the higher concentration of the monomeric form, which in the case of AMB is non-toxic for mammalian cells.

The self-association of AMB in solutions has usually been followed by spectroscopic methods: UV-Vis and circular dichroism (CD). The spectroscopic properties of AMB are determined by the presence of the *all-trans* heptaenic chromophore. UV-Vis spectra of the monomeric form of AMB in the range of 300–450 nm are characterized by four bands, whose intensities and positions are solvent dependent. AMB in polar solvents like methanol, ethanol and DMSO exists as a monomolecular solution and its optical absorption spectra are not concentration dependent. For example, the spectrum of AMB in methanol is characterized by bands at 407, 383, 364, 346 nm. In aqueous solutions of AMB, in which self-association occurs, the optical absorption spectra of such solutions are concentration dependent. At a very low concentration of AMB below 0.1 μM the spectrum is similar to that in polar solvents, with small red shifts. The band positions in the spectra of such antibiotic solutions are 409, 384, 365 and 348 nm. As the antibiotic concentration increases the spectrum is progressively modified, and finally, at the concentration of 100 μM, when practically all the AMB molecules are aggregated, a new spectrum is observed. In pure water this spectrum is characterized by a new wide band with a maximum at 339 nm, and two minor peaks at 390 and 420 nm [12]. It was shown that substitutions at the polar head of the molecule had no effect on spectral properties of the polyenic chromophore [1]. Consequently, the same spectroscopic methods can be applied to study the AMB derivatives. The MFAME self-association process has not been systematically studied in aqueous media so far. However, it was observed that its aspartate salt is well soluble in water and glucose solutions, but in PBS it formed insoluble aggregates at relatively low concentrations. It should be noted that most of the experiments on erythrocytes have been performed in PBS thus far, meaning that the observed low haemolytic activity of MFAME in this medium could be underestimated. The results presented in this study indicate that the MFAME self-association is strongly influenced by medium composition.The purpose of this work was to examine:

- self-association of MFAME molecules in aqueous solutions and the influence of medium composition on this process;
- the relationship between the MFAME self-association and its effectiveness towards red blood cells.

The self-association process has been followed by measurement of the electronic absorption in the UV-Vis region as a function of antibiotic concentration. A principle component analysis (PCA) was applied to find out how many species co-exist in aqueous solutions of MFAME. The effectiveness on RBC was measured by determination of the potassium efflux and extent of haemolysis.

2. Materials and methods

2.1. Polyene antibiotics

AMB was from Sigma. MFAME and its L-aspartate salt were synthesized in our laboratory according to the previously described method [6]. In all determinations, water-soluble MFAME-L-aspartate was used, which in the text is abbreviated as MFAME. Purity of the substances was determined spectrophotometrically. ($\varepsilon = 160\,000\ M^{-1}\,cm^{-1}$ for methanolic solutions of AMB and its derivative). All concentrations given in the text are calculated for 100% pure compounds.

2.2. Determination of the proportions of antibiotic species in aqueous solutions

2.2.1. Sedimentation method

Stock solutions of MFAME were prepared in water, just before use, in concentration 100-fold higher than the desired final one. The final solutions were obtained by adding a proper volume of stock solution to water or following aqueous media: 155 mM sodium chloride (saline), 155 mM sodium chloride buffered with 7 mM sodium phosphate pH 7.4 (PBS), 300 mM glucose, 300 mM sucrose, and 300 mM glucose buffered with 7 mM sodium phosphate pH 7.4. After 1 h of incubation at room temperature, solutions containing different concentrations of MFAME were divided into two samples: (a) and (b). Absorption spectra of the sample (a) and sample (a) diluted with methanol 1:1 (a′) were recorded. The sample (b) was centrifuged at $12\,200 \times g$ for 10 min and absorption spectra of the supernatant of sample (b) and the supernatant diluted with methanol 1:1 (b′) were recorded as well. Spectroscopic measurements in the UV-Vis range 300–450 nm was done with Cary 300 (Varian) spectrophotometer.

Antibiotic concentrations were determined according to the molar absorption coefficient at 409 nm in the case of aqueous solutions and at 408 nm in the case of aqueous/methanol mixtures.

Total antibiotic concentration (C_t) was determined in sample (a′), monomer concentration (C_m) in sample (b), concentration of soluble forms, monomer and soluble aggregates (C_m+C_{sa}), in sample (b′). Concentration of soluble aggregates (C_{sa}) and insoluble aggregates (C_{is}) was determined according to the formulae:

$$C_{sa} = (C_m + C_{sa}) - C_m$$

$$C_{is} = C_t - (C_m + C_{sa})$$

For each medium and antibiotic concentration the experiments were repeated at least three times.

As molar absorption coefficient ε_m is dependent on solvent polarity it was determined in aqueous media and methanol/aqueous solution mixture. The molar absorption of the sample (a) and sample (a′) at λ_{408} and λ_{409} as a function of antibiotic concentration was plotted and ε_m was obtained by extrapolation of antibiotic concentration to 0; aqueous medium composition had no influence on ε_m value, being 101 500 $M^{-1}\,cm^{-1}$ for aqueous solutions of MFAME and 124 000 $M^{-1}\,cm^{-1}$ for 1:1 v/v methanol/aqueous solution mixtures.

Wavelengths of 408 and 409 nm were chosen because at these wavelengths there was a great difference between the absorption of monomeric and aggregated forms of antibiotic. In fact, the absorption at these wavelengths reflect the concentration of the monomeric form of the antibiotic.

2.2.2. Numerical decomposition of UV-Vis spectra

The absorption spectrum in the range 300–450 nm has been registered in numerical form with wavelength steps of 1 nm, recalculated to molar extinction values and stored as a set of 151 real numbers. A total of $n = 90$ such spectra determined in 300 mM glucose, saline, and PBS for a range of MFAME concentration 1–120 μM formed the rectangular matrix **S** consisting of 151 rows (wavelengths) and 90 columns (spectra). This matrix was an object of principal component analysis (PCA) and numerical spectrum decomposition (NSD).

2.3. Principal component analysis [14]

The acquired spectra matrix **S** was factor analysed in order to estimate the number of independent components (spectral species) giving rise to the acquired data. The purpose of factor analysis is to provide a representation of the spectra in a space with significantly smaller dimensionality. This was achieved by taking into account the correlation between each pair of spectra. To calculate the correlation matrix between the spectra, the values of each spectrum were first normalized according to formula:

$$w_{ij} = \frac{s_{ij} - \mu_j}{\sigma_j} \tag{1}$$

where μ_j is the mean of the values of the jth spectrum, σ_j is the standard deviation of the jth spectrum, s_{ij} is the extinction coefficient at ith wavelength of jth spectrum, forming normalized matrix **W**. The correlation matrix **C** was given as

$$\mathbf{C} = \frac{1}{n-1}\mathbf{W}^{T}\mathbf{W} \tag{2}$$

The principal component decomposition of the correlation matrix **C** formed n orthogonal eigenvectors. Associated with each eigenvector is a descriptor of its importance called eigenvalue. The minimum number of eigenvectors (those with the largest eigenvalue) needed to account for the variations in the data define the number of independent spectral species, m. Because of the experimental noise, the first m eigenvectors may not account for 100% of the spectra variance. In practice, slightly smaller values are found. After VARIMAX rotation and renormalization the eigenvectors might be used as a first approximation of components spectra.

2.4. Numerical spectrum decomposition [15]

If acquired spectra obey Beer's law the spectral matrix **S** might be expressed as a product of two other matrices: a component matrix **K**, columns of which represent spectra of pure spectral species, and molar ratio matrix **X**, columns of which contain molar ratios of spectral species present in each sample.

$$\mathbf{S} = \mathbf{KX} \tag{3}$$

The number of independent spectral species as well as the

first approximation of their spectra might be estimated by PCA. However, the eigenvectors are obtained with the assumption that they are perfectly orthogonal. This assumption is not true for spectra of the species. Thus, to solve Eq. 3 an iterative self-consisting procedure called numerical spectrum decomposition (NSD) was employed:

(1) The linear combination of the rotated and renormalized eigenvectors were used to obtain a first approximation of columns of the matrix **K** (spectra of pure species). The eigenvectors were combined manually to obey two rules: (i) spectra should contain positive values only; (ii) the number, position and intensities of absorption bands in component spectra should be similar to the same features in spectra of representative samples.

(2) Approximation of matrix **K** was used to calculate molar ratio matrix **X** according to the equation:

$$\mathbf{X} = [\mathbf{K}^{\mathrm{T}}\mathbf{K}]^{-1}\,\mathbf{K}^{\mathrm{T}}\mathbf{S} \tag{4}$$

(3) The matrix **X** was corrected to obey two rules: (i) the molar ratios should not be negative; (ii) the sum of molar ratios for each sample must be equal to 1. To obey the rules negative values in **X** were set to 0 and each column of **X** was rescaled forming the matrix **Y**.

(4) The matrix **Y** was used to calculate new approximation of component matrix **K** according to a formula:

$$\boldsymbol{K} = \boldsymbol{S}\boldsymbol{Y}^{T}[\boldsymbol{Y}\boldsymbol{Y}^{T}]^{-1} \tag{5}$$

(5) The matrix **Y** from step (3) and the matrix **K** from step (4) were used to recalculate the spectral matrix **V**:

$$\mathbf{V} = \mathbf{K}\mathbf{Y} \tag{6}$$

Differences between elements of matrixes **S** and **V** were used to calculate root mean square error, RMSE, between experimental and recalculated spectra. If RMSE values for two subsequent iterations were the same, the procedure was stopped. In opposite cases the iteration was restarted from step (2) with matrix **K** obtained in step (4).

2.4.1. Erythrocytes

Human blood, citrate anticoagulated, was kept at 4°C. Just before use, erythrocytes were separated by centrifugation (10 min, 3000 rpm, 4°C) and were washed three times with 155 mM sodium chloride (saline).

2.5. Permeabilizing activity and extent of haemolysis

Aqueous solutions of MFAME or AMB were incubated for 1 h at room temperature and then erythrocytes (final concentration 10^8 cells/ml) were added.

After 1 h of incubation with the antibiotic, the amount of potassium released into the medium was determined with a potassium-selective electrode (Radiometer Copenhagen). The degree of haemolysis was determined after 1, 3, 6 and 24 h exposure of the cells to antibiotic. The samples were centrifuged and the absorbance of the supernatant diluted 10-fold with water was measured at 540 nm with a spectral colorimeter. The values for 100% of haemolysis and potassium release were obtained in sample haemolysed in water.

3. Results

3.1. Self-association of MFAME in aqueous solutions

Absorption spectra of 120 μM MFAME in methanol, 300 mM glucose, saline and PBS are compared in Fig. 2. Spectrum of MFAME in methanol is the same as a spectrum of the monomeric form of AMB with four bands at 407, 385, 364 and 346 nm. The spectra of aqueous solu-

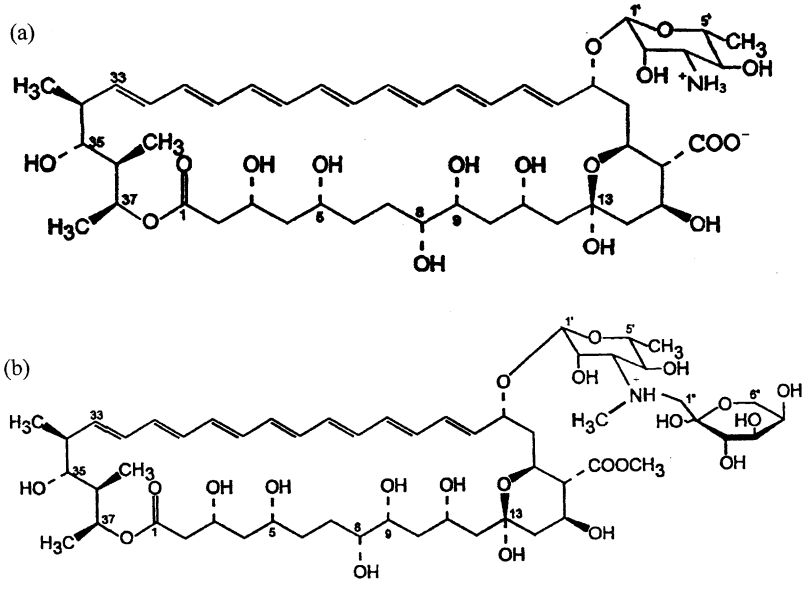

Fig. 1. Structures of (a) AMB and (b) MFAME.

tions of antibiotic are modified and indicate that MFAME undergoes self-association. The absorption spectra of MFAME in saline and in PBS are similar in the respect to the shape. The characteristic features of these spectra are disappearance of the band characteristic for the monomeric form at 409 nm, and appearance of new bands, one at 420 nm and a second wide band with a maximum at 345 nm, which in fact replace the absorption of the monomeric form. The lower spectrum intensity in PBS than in saline is probably due to precipitation of insoluble aggregates in the first medium. The absorption spectra of MFAME in 300 mM sucrose, 300 mM glucose and pure water were the same, but different from those in PBS and saline. The spectrum of MFAME in 300 mM glucose is shown as an example. In this spectrum three bands characteristic for monomer of MFAME at 409, 384 and 365 nm can be recognized, but their intensities and general spectrum shape are different. The wide band with a maximum at 365 nm is dominant. The main differences in the spectra of antibiotic in 300 mM glucose and salt solutions are that the band at 409 nm considered as characteristic for the monomeric form is present only in glucose solutions, one of the bands indicating self-association at 420 nm is significantly weaker in glucose medium than in saline and PBS, and the other, at the shorter wavelength, has a different position. We can state that MFAME exhibits a tendency towards aggregation in all examined aqueous media. UV-Vis spectra indicate that in these media MFAME is present in monomeric form in concentrations below 1 μM. This means that MFAME self-association begins in concentration 10-fold higher than in the case of AMB. The result also suggests a lower tendency of MFAME to self-association in pure water and sugar-containing media than in mineral salt solutions, and lead to suppose that antibiotic forms aggregates with different spectroscopic properties depending on medium composition.

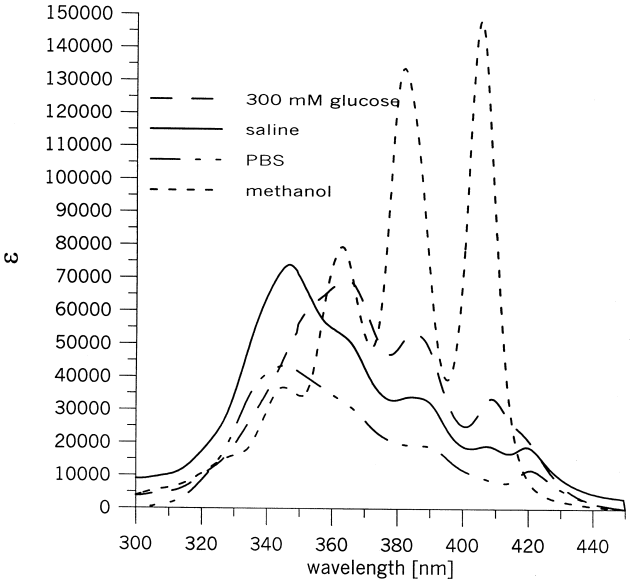

Fig. 2. Absorption spectra of 120 μM MFAME in methanol and aqueous media. Spectroscopic measurements of solutions were done after 1 h of incubation at room temperature.

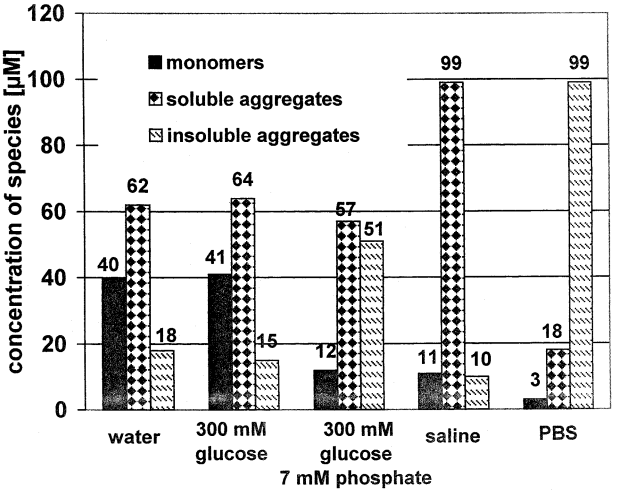

Fig. 3. Concentration of particular MFAME species in various aqueous media determined by sedimentation method; total antibiotic concentration 120 μM.

3.2. Proportions of antibiotic species in various aqueous solutions

The proportions of antibiotic species in different media were determined by the sedimentation method. Soluble species monomer and aggregates were separated from insoluble ones by centrifugation. The estimation of the proportions of monomer and aggregates was based on an assumption that in aqueous solutions absorption at 409 nm reflected only the monomer concentration. Monomer concentration C_m was determined according to the molar absorption coefficient at 409 nm in aqueous solutions. Total antibiotic concentration C_t as well as soluble forms concentration, monomer and aggregates (C_m+C_{sa}), were determined in aqueous/methanol mixtures, in which antibiotic exists in monomeric form. Concentration of soluble aggregates (C_{sa}), and insoluble aggregates (C_{is}) was obtained according to the formulae given in Section 2.

In Fig. 3 the amounts of particular antibiotic species in aqueous solutions for total antibiotic concentration 120 μM are presented. The highest monomer concentration, about 40 μM, was detected in water, 300 mM sucrose and 300 mM glucose. In mineral salt-containing media, monomer concentrations were significantly diminished on the account of self-associated species. The amount of soluble aggregates was higher in saline than in 300 mM sucrose, 300 mM glucose and water. In contrast, in PBS concentration of soluble species was very low. In PBS and 300 mM glucose with 7 mM sodium phosphate, concentration of insoluble aggregates was much higher than in media which did not contain sodium phosphate. The presence of sodium phosphate in glucose medium as well as in saline increased the tendency of antibiotic to form

insoluble aggregates, which can be separated by centrifugation.

The proportions of monomer, soluble oligomers, and insoluble aggregates as a function of MFAME concentrations in 300 mM glucose, saline and PBS were determined. The results are presented in Fig. 4A–C. In the concentration range studied, monomer concentrations are significantly higher in 300 mM glucose (Fig. 4A) than in mineral salt-containing media (Fig. 4B,C). At antibiotic concentrations below 60 μM in 300 mM glucose solutions monomer prevails, and above 60 μM soluble aggregates predominate (Fig. 4A). In saline the amount of soluble aggregates increases monotonically with total antibiotic concentration and prevails in the concentration range examined (Fig. 4B). It is much higher than in 300 mM glucose.

Insoluble aggregates are formed mainly in PBS, while in 300 mM glucose and saline the concentration of insoluble forms is significantly lower. In PBS, concentration of insoluble aggregates increased dramatically with total antibiotic concentration (Fig. 4C).

As we have shown in Fig. 2, the spectra of MFAME solutions are characterized by different shape and band positions dependent on medium composition. This fact suggests that MFAME molecules might form several types of aggregates with different spectroscopical properties. In order to find out how many species co-exist in aqueous solutions of MFAME we applied a principal component analysis (PCA). Four independent spectral components have been detected by this method in the set of MFAME spectra obtained at a concentration range between 5 and 120 μM in different aqueous media. These components explain 99.55% of the total variance included in analysed spectra. Numerical decomposition of the spectra based on these components provided spectra of the four spectral species Fig. 5. One of them exhibits spectral features similar to that of MFAME spectrum in methanol and thus might be ascribed to the monomeric form of the compound in aqueous environment. The other three species are characterized by the spectra typical rather for aggregated forms of AMB. The spectrum of species called by us type I exhibits absorption bands at wavelengths similar to those of the monomer. However, contrary to the monomer, the intensity of band at 409 nm is low and bands at 365 and 355 nm are the most intensive.

In a spectrum of aggregates the low absorption maximum at 409 nm in type II, similar to the spectrum of aggregates type I, is observed. The most intensive band in the spectrum of aggregates type II is localized at 327 nm. The spectrum of aggregates type III is characterized by absorption maxima at 420 and 345 nm. The characteristic features of this spectrum is lack of absorption maximum at 409 nm, which is present in the spectra of monomeric form, as well as of aggregates type I and II.

Molar ratios of each species in all samples studied were also obtained as a result of the numerical decomposition of the spectra. These ratios can be used to calculate con-

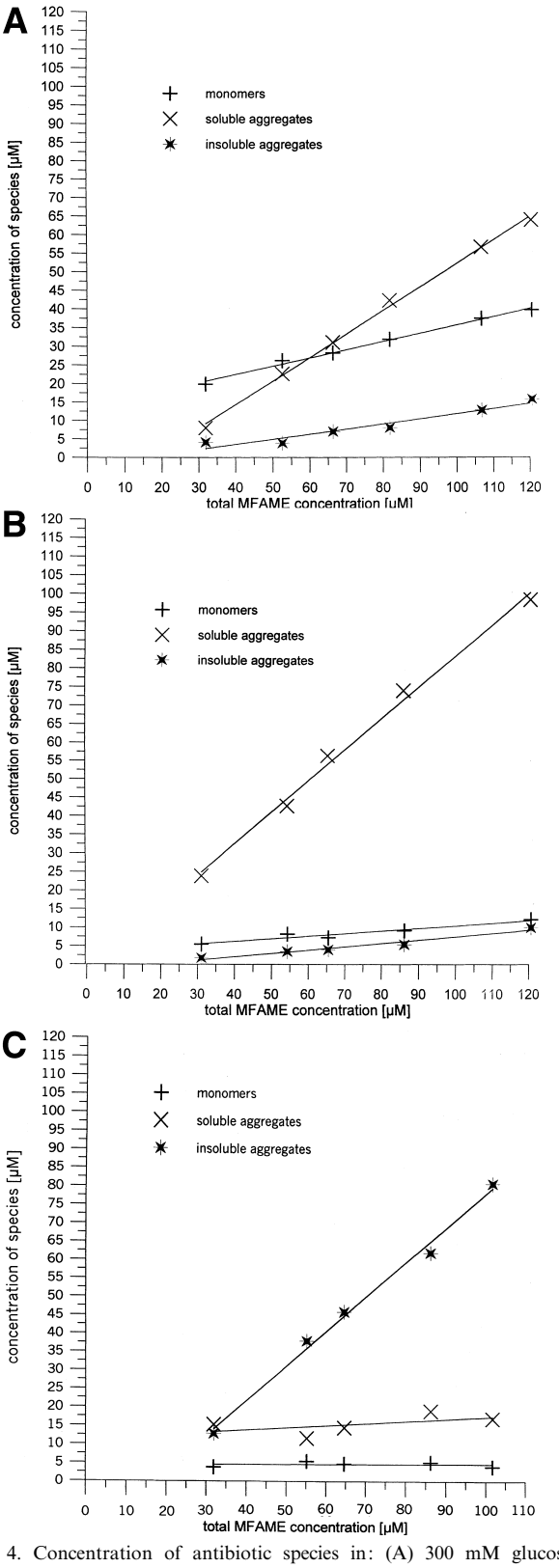

Fig. 4. Concentration of antibiotic species in: (A) 300 mM glucose; (B) saline; (C) PBS as a function of total antibiotic concentration, determined by sedimentation method.

centrations of monomer and aggregated species. Changes in the concentration of the particular species with increasing total concentration of MFAME in different media are presented in Fig. 6. In all solutions MFAME exists in aggregated form, and monomer concentrations are relatively low. In glucose-containing media the antibiotic forms mainly the aggregates type I (Fig. 6A), while in saline (Fig. 6B) and PBS (Fig. 6C) aggregates type III prevails. The aggregates type II are present in saline, in total antibiotic concentrations below 20 μM.

Comparing the results obtained from the sedimentation method and from numerical analysis we can state that:

- The medium composition is a main factor determining which type of aggregates prevail in MFAME solutions.
- Antibiotic behaviour is quite different in phosphate-containing media than in media which do not contain phosphate anions. This difference concerns solubility of the compound. In mineral salt-containing media MFAME exists mainly in a form of aggregates type III in wide concentration range. The sedimentation method indicates that these aggregates are soluble in saline solutions, while in the presence of phosphate they precipitate.
- In 300 mM glucose soluble aggregates type I prevail. The amount of monomers determined according to absorption coefficient at 409 nm seems to be overestimated. This is due to spectroscopic properties of aggregates type I which have an absorption at 409 nm.

3.3. Effect of MFAME on red blood cells

Effectiveness of MFAME on red blood cells was measured by determination of intracellular potassium efflux and haemolytic activity in 300 mM sucrose, saline, PBS. As has been shown, in these media type and concentration

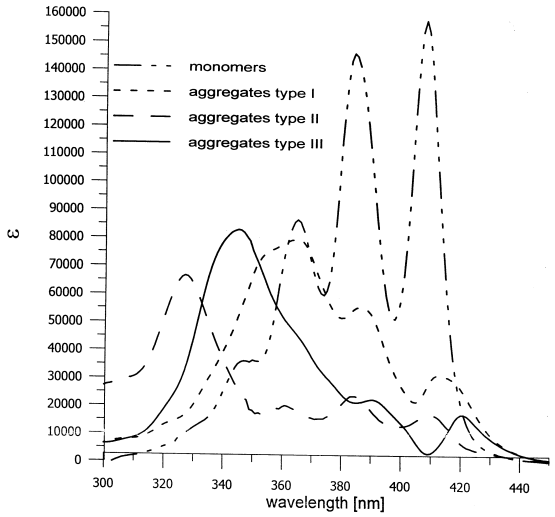

Fig. 5. Absorption spectra of the MFAME species present in aqueous solutions obtained from numerical analysis.

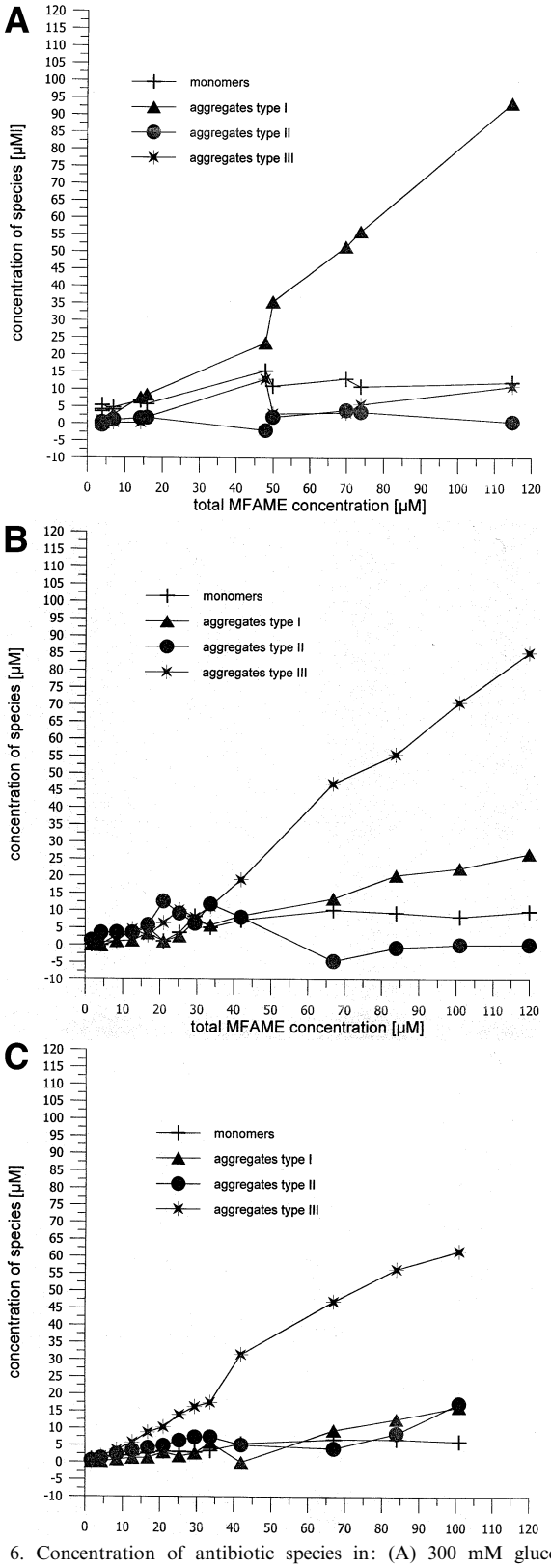

Fig. 6. Concentration of antibiotic species in: (A) 300 mM glucose; (B) saline; (C) PBS as a function of total antibiotic concentration obtained by numerical analysis.

of soluble species are different. It was impossible to measure antibiotic activity in 300 mM glucose due to cell haemolysis. The lowest permeabilizing activity MFAME occurs in PBS in which the compound exists mainly in the form of insoluble aggregates (Fig. 7). In saline and sucrose solutions the effectiveness of MFAME is much higher than in PBS, suggesting that soluble aggregates are the active form of MFAME in red blood cells. The differences in the permeabilizing activity of MFAME in saline and sucrose are not so significant as could be expected on the basis of differences in the concentration of soluble aggregates in these media determined by the sedimentary method. In sucrose solutions in the presence of MFAME, haemolysis is not observed, indicating that MFAME, similar to parent AMB, induces membrane damage not permeable to sucrose molecules. Haemolytic activity of MFAME (Fig. 8) is observed in saline solutions. In this medium concentration of soluble oligomers increases with total antibiotic concentration and haemolytic activity of MFAME increases in the same way. In PBS solutions in the antibiotic concentration range studied, in which the amount of soluble forms are very low, MFAME is practically not haemolytic.

Taking into account the results of numerical analysis of the spectra, it seems that the active forms of MFAME are aggregates type I and III, which prevail in sucrose and saline, respectively. However, type III aggregates are ineffective in PBS, because in these media they precipitate. It should be stressed that MFAME aggregates are characterized by much lower efficiency towards red blood cells than AMB. At a concentration of 120 μM in saline, in which concentration of soluble aggregates is about 100 μM, MFAME induced about 50% potassium efflux from erythrocytes in 1 h, while AMB at a concentration of 5 μM under the same experimental conditions caused almost

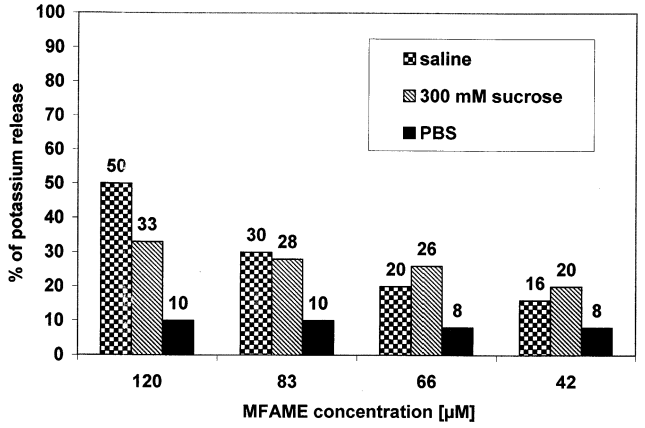

Fig. 7. Potassium released from red blood cells by MFAME in sucrose, saline and PBS. Aqueous solutions of MFAME were pre-incubated for 1 h at room temperature and then erythrocytes (final concentration 10^8 cells/ml) were added. The amount of potassium released into the medium was determined with potassium-selective electrode, after 1 h of incubation with antibiotic.

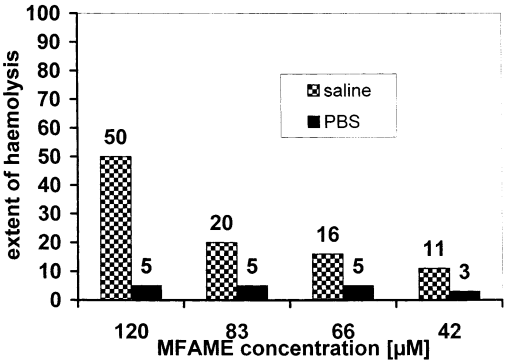

Fig. 8. Extent of haemolysis induced by MFAME in saline and PBS. Aqueous solutions of MFAME were pre-incubated for 1 h at room temperature and then erythrocytes (final concentration 10^8 cells/ml) were added. Degree of haemolysis was determined after 24 h exposure of the cells to antibiotic.

100% potassium efflux in 10 min, and complete haemolysis in 2 h (data not shown).

4. Discussion

The understanding of the mechanism of biological activity of AMB and especially of its selectivity for ergosterol- and cholesterol-containing cells is of primary importance for the development of new AMB derivatives of low toxicity. It is generally accepted that the toxic effect of AMB is a consequence of increased membrane permeability due to formation of specific structures (permeabilizing species) as a result of the antibiotic interaction with membrane located sterols (reviewed in [7]). Many experimental and theoretical studies which have been performed to recognize the molecular basis of the selectivity of AMB suggest that it is rather a multifactorial phenomenon. Several molecular factors such as quantitative and qualitative differences in the interaction of the compound with ergosterol and cholesterol, stability of the channel formed in membrane of fungal and mammalian cells, selectivity of the permeabilizing pathway, conformational properties and state of the antibiotic in solution, are considered to be important for selective toxicity of AMB.

The differences in the affinity of AMB to cholesterol and ergosterol could be modulated by the chemical modification on the 'polar head' of the antibiotic. The extent of interaction with sterols was correlated with the biological activity of compounds tested [16,20]. The analysis of the distribution of the molecular electrostatic potential (MEP) of the antibiotic and sterol molecules allowed to put forward the hypothesis of the differential affinity of the antibiotic to the ergosterol and cholesterol. It was shown that there are well-defined differences between electrostatic patterns of the cholesterol and ergosterol molecules in the area of the side chain. These different patterns may be responsible for experimentally observed higher affinity of

AMB for ergosterol than for cholesterol [2,17]. The interactions responsible for the formation and stability of the primary AMB–sterol complexes were recognized. The main forces binding both complex components are the Van der Waals interactions, which are similar for both sterols. However, for the stability of the complex the orienting forces are essential. To properly orient the complex constituents, the presence of at least two attachment points located at a proper distance is indispensable. One of them is a hydrogen bond network generated by the protonated amino group of the amino sugar moiety and carboxyl group of AMB in which the OH group of sterols participate. The second attachment point, stabilizing the complex, is an interaction between the antibiotic chromophore and the ergosterol side chain in which C=C double bond is a source of negative potential. This interaction is lacking in the case of the cholesterol where such a negative area at the side chain is not present [2,18].

Stability of the channel formed by AMB in cholesterol- and ergosterol-containing membrane seems to be another factor influencing selectivity of AMB and its derivatives. A molecular dynamics simulation for a model of the AMB membrane channel revealed the important role of the intermolecular interactions among the hydroxyl, amino, and carboxyl groups of the channel-forming molecules [19]. On the basis of this simulation the experimental data concerning biological activity and selectivity of AMB derivatives with the modified amino and carboxyl group could be explained [20]. For AMB derivatives with altered amino and/or carboxyl group, hydrogen bonds formed are not so strong as the bonds network formed between free amino and carboxyl groups of the neighbouring AMB molecules. These weaker hydrogen bonds probably make the structure of the AMB/cholesterol channel less stable than the AMB/ergosterol channel that result in the improved selectivity [19].

The influence of the conformational factors of AMB derivatives on selective toxicity was also examined [21]. The conformational analysis of AMB amide derivatives together with the results of dynamics simulation of AMB membrane channels allowed building of a new model of structure-selective toxicity of AMB derivatives. In this proposed model the presence of the extended conformation as the only well-defined global conformer for AMB derivatives is taken as the indicator of their higher selective toxicity [19,21].

The role of another factor, the channel selectivity in the selective toxicity of AMB, has not as yet been explained [22].

The studies on the action of AMB on ergosterol- and cholesterol-containing membranes and cells [8–11] showed the important role of the degree of AMB self-association in selective toxicity. The soluble aggregates were active on cholesterol- and ergosterol-containing LUVs and cells, while monomers were effective only on ergosterol-containing membranes. The amphiphilic and amphoteric character of AMB molecule provoke antibiotic self-association in aqueous media. On the basis of experiments concerning the influence of the ionic state of the AMB molecule on the aggregation behaviour a multi-step model of polyene antibiotic self-association was proposed in which dimerization of monomers to more hydrophilic dimers is a first step. It seems that the chemical modification which diminishes or destroys ability of the dimer formation results in increasing AMB selective toxicity [24]. It was demonstrated that the net electric charge of the antibiotic molecule is an important factor influencing the aggregation and the solubility of AMB and its derivatives. The net charge on the molecule increases solubility in two ways: first, by decreasing the dimerization and association constants, and secondly, by increasing the threshold of the concentration for which oligomers are water soluble [23].

All of these results brought forward a clue for the rational design of AMB derivatives with improved pharmacological properties. The designed compounds should have (i) the bulky substituents at the amino group that should still be able to form weak hydrogen bonds; (ii) the substitution at the carboxyl group [19,20]; and (iii) the net charge [23].

N-Methyl-*N*-D-fructosyl amphotericin B methyl ester (MFAME) is a compound designed according to this strategy (Fig. 1). Such chemical modification of AMB molecule, according to our expectation, did not affect significantly its activity towards fungal cells, and resulted in the much higher selective toxicity in comparison with the parent antibiotic. At the same time the radical improvement of its solubility in aqueous media was achieved [4–6]. The studies aimed to explain which properties of the new derivative determine its low toxicity towards cholesterol-containing cells. Comparative studies of MFAME 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 these results it was proposed that the improvement of selective toxicity of MFAME could in part be a consequence of the diminished concentration of water-soluble oligomers, as well as better ability to differentiate between the ergosterol and cholesterol [1]. The experiments have shown that for the monomeric form of MFAME, the discrimination between the ergosterol and cholesterol was more pronounced than for AMB. However, this difference was not sufficient to explain the dramatic improvement of selective toxicity of the derivative [1]. This means that the role of the quantitative differences in the interaction of MFAME with both sterols is not so important for its selective toxicity as it was for AMB and some other derivatives. The present work is a continuation of the study on the influence of MFAME self-association on its toxicity towards cholesterol-containing cells. We have compared the activity of the self-associated form of MFAME to-

wards red blood cells in PBS, 300 mM sucrose and saline. It has been shown that the activity of MFAME depends on the state of antibiotic in solution. A very low activity of MFAME in PBS, and significantly higher activity in saline and sucrose solution, indicate that insoluble aggregates of MFAME are an inactive form of the antibiotic. Monomers are also inactive, because in concentrations in which the compound exists in monomeric form the activity towards erythrocytes is not observed. The active form of MFAME towards cholesterol-containing red blood cells, similar to the case of AMB, are soluble aggregates. However, the efficiency of the aggregates formed by MFAME is much lower than that formed by the parent antibiotic. The extent of membrane permeabilization induced in red blood cells by MFAME never reached the level induced by AMB aggregates even if the concentration of the derivative was two orders of magnitude higher. Differences in CD spectra of aggregates of MFAME and AMB suggest that they have a different structure [1]. It seems that the structure of aggregates is an important factor influencing the efficiency of the compound. The role of other factors such as qualitative differences in the interaction with sterols, structure and properties of the channel formed in membranes of fungal and mammalian cells, which probably also decide about the high selective toxicity of MFAME, has not been studied thus far. The aim of our further experimental and theoretical work will be to investigate the importance of these factors for selective toxicity of new compounds. Unfortunately, the theoretical studies on the properties of MFAME are not possible now, because detailed geometry of the molecule is not yet known. However, the calculation can be performed for other AMB derivatives like Orn-AME, characterized by similar properties as MFAME, for which indispensable data are available.

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References

[1] B. Cybulska, I. Gadomska, J. Mazerski, J. Grzybowska, E. Borowski, M. Cheron, J. Bolard, Acta Biochim. Pol. 47 (2000) 121–131.
[2] J. Mazerski, E. Borowski, Task Q. (Gdansk) 2 (1998) 511–550.
[3] E. Borowski, Il Farmaco 55 (2000) 206–208.
[4] J. Szlinder-Richert, B. Cybulska, J. Grzybowska, R. Prasad, E. Borowski, Acta Biochim. Pol. 47 (2000) 133–140.
[5] M.M. Bontemps-Gracz, J. Grzybowska, R. Supino, E. Borowski, Abstracts of 7th International Symposium on Molecular Aspects of Chemotherapy, Gdańsk, Poland, September, 1999, p. 159
[6] J. Grzybowska, P. Sowiński, J. Gumieniak, T. Zieniawa, E. Borowski, J. Antibiot. 50 (1997) 709–711.
[7] J. Bolard, Biochim. Biophys. Acta 864 (1986) 257.
[8] J. Bolard, P. Legrand, F. Heitz, B. Cybulska, Biochemistry 30 (1991) 5707–5715.
[9] P. Legrand, E.A. Romero, B.E. Cohen, J. Bolard, Antimicrob. Agents Chemother. 36 (1992) 2518–2522.
[10] J. Barwicz, P. Tancrede, Chem. Phys. Lipids 85 (1997) 145–155.
[11] K. Yamashita, V. Janout, E.M. Bernard, D. Armstrong, S. Regen, J. Am. Chem. Soc. 117 (1995) 6249–6253.
[12] F. Gaboriau, M. Cheron, L. Leroy, J. Bolard, Biophys. Chem. 66 (1997) 1–12.
[13] I. Gadomska, B. Cybulska, J. Grzybowska, J. Bolard, E. Borowski, Abstracts of 5th International Symposium on Molecular Aspects of Chemotherapy, Gdańsk, Poland, August, 1995, p. 100.
[14] M.A. Sharaf, D.L. Illman, B.R. Kowalski, Chemometrics, Wiley, New York, 1986.
[15] R. Kramer, Chemometric Techniques for Quantitative Analysis, Marcel Dekker, New York, 1998.
[16] J. Mazerski, J. Bolard, E. Borowski, Biochim. Biophys. Acta 1236 (1995) 170–176.
[17] M. Baginski, P. Bruni, E. Borowski, Theochem. J. Mol. Struct. 311 (1994) 285–296.
[18] M. Baginski, E. Borowski, Theochem. J. Mol. Struct. 389 (1997) 139–146.
[19] M. Baginski, H. Resat, J. Andrew Mccammon, Mol. Pharmacol. 52 (1997) 560–570.
[20] M. Cheron, B. Cybulska, J. Mazerski, J. Grzybowska, A. Czerwiński, E. Borowski, Biochem. Pharmacol. 37 (1988) 827–836.
[21] H. Resat, F. Aylin Sungur, M. Baginski, E. Borowski, V. Aviyente, J. Comput.-Aided Mol. Des. 14 (2000) 689–703.
[22] B. Cybulska, J. Bolard, O. Seksek, A. Czerwinski, E. Borowski, Biochim. Biophys. Acta 1240 (1995) 167–178.
[23] J. Mazerski, J. Grzybowska, E. Borowski, Eur. Biophys. J. 18 (1990) 159–164.
[24] J. Mazerski, E. Borowski, Biophys. Chem. 57 (1996) 205–217.