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QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS IN AMPHOTERICIN B DERIVATIVES

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Abstract—The quantitative structure–activity relationships studies of amphotericin B and its 16 semi-synthetic derivatives obtained by modification at carboxyl and amino groups have been done. The results of five biological tests were subjected to principal component analysis, a numerical method useful in the investigation of large sets of data. For some compounds, also, interaction with lipidic vesicles was investigated by spectroscopic methods. The results obtained indicate that:

(i) The presence of positively charged nitrogen atom (protonable or bearing fixed charge) is indispensable for biological activity and antibiotic–sterol interaction;

(ii) The lack of free carboxyl group in the molecule favours the differentiation between cholesterol and ergosterol containing cells.

The lack of an effective and non-toxic drug for the treatment of systemic mycoses still remains the major problem in antifungal chemotherapy. The most effective drug, amphotericin B, a polyene macrolide antibiotic, causes serious side effects such as nephrotoxicity, haemolytic anemia and electrolyte abnormalities [1]. Low selective toxicity of amphotericin B results from the same molecular basis of the toxic action towards mammalian and fungal cells. Action of amphotericin B and of all others polyene macrolides is located at the level of plasma membrane and restricted to sterol containing eucariotic cells. Polyene macrolides interacting with sterols located at the plasma membrane of sensitive cells cause impairment of barrier function, leakage of cellular constituents and ultimately cell death. It has been recently evidenced that in fungus-invaded organisms the action of amphotericin B is not restricted to the lethal changes of membrane permeability in pathogenic cells but also can be augmented by stimulation of both humoral and cell immunity (for review see Refs. 2 and 3).

There is much evidence that cell sensitivity to polyene macrolides is determined by sterol composition of the plasma membrane. The quantitative difference in the relative affinities of ergosterol-containing fungal membranes and cholesterol-containing mammalian membranes for these antibiotics seems to be the major determinant of the therapeutic effect of amphotericin B. A better recognition of the structural features of polyene macrolide antibiotics responsible for differentiation between cholesterol- and ergosterol-containing cells would give a basis for rational design of new compounds characterized by improved pharmacological properties.

In the present work the effect of structural changes in amphotericin B molecule on biological activity and selective toxicity has been studied by the principal component analysis (PCA)—one of the methods of the quantitative structure–activity relationship analysis (QSAR) [4, 5].

A series of amphotericin B derivatives has been obtained by modification of the ionizable polar groups comprising carboxyl attached to the macrolide ring and amino group of the amino-sugar moiety. The amino group was modified by acetylation, aminoacylation or alkylation, whereas the carboxyl group was modified by esterification or amidation [6]. Structures of the examined compounds are shown in Fig. 1. Such a set of compounds allowed us to determine the significance of following structural features for the activity and selective toxicity:

(i) net charge of the molecule;

(ii) presence of free carboxyl group or type of its modification;

(iii) presence and mobility of protons covalently bound to nitrogen atom of amino-sugar moiety;

(iv) position of the positively charged nitrogen atom.

Biological properties of the compounds were tested on *Saccharomyces cerevisiae* and *Candida albicans* used as models of ergosterol-containing pathogenic cells and on human red blood cells, representative of cholesterol-containing host cells. The activity tests applied were: yeasts growth inhibition, potassium release from yeast and erythrocytes, and haemolysis of erythrocytes (Table 1). For some compounds, also, their interaction with cholesterol- and ergosterol-containing large unilamellar lipidic vesicles was examined by spectroscopic methods.

MATERIALS AND METHODS

Amphotericin B and its derivatives. Amphotericin B was from Squibb & Sons (Princeton, NJ) and was used without further purification. All amphotericin

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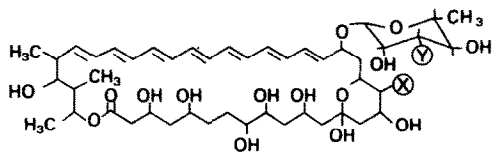

No	Compound	X	Y
1	amphotericin B AMB	COO^-	$\overset{+}{N}H_3$
2	amphotericin B methyl ester AME	$COOCH_3$	$\overset{+}{N}H_3$
3	amphotericin B 3-(N', N'-dimethylamino) propyl amide AMA	$CONH(CH_2)_3\overset{+}{N}H(CH_3)_2$	$\overset{+}{N}H_3$
4	N-acetylamphotericin B Ac-AMB	COO^-	$NHCOCH_3$
5	N-acetylamphotericin B methyl ester Ac-AME	$COOCH_3$	$NHCOCH_3$
6	N-acetylamphotericin B 3-(N', N'-dimethylamino) propyl amide Ac-AMA	$CONH(CH_2)_3\overset{+}{N}H(CH_3)_2$	$NHCOCH_3$
7	N-glycylamphotericin B methyl ester Gly-AME	$COOCH_3$	$NHCOCH_2\overset{+}{N}H_3$
8	N-(N', N'-dimethylglycyl) amphotericin B DMGly-AMB	COO^-	$NHCOCH_2\overset{+}{N}H(CH_3)_2$
9	N-(N', N'-dimethylglycyl) amphotericin B methyl ester DMGly-AME	$COOCH_3$	$NHCOCH_2\overset{+}{N}H(CH_3)_2$
10	N-(N', N'-dimethylglycyl) amphotericin B 3-(N',N'-dimethylamino) propyl amide DMGly-AMA	$CONH(CH_2)_3\overset{+}{N}H(CH_3)_2$	$NHCOCH_2\overset{+}{N}H(CH_3)_2$
11	N-(N', N'-dimethyl-β-alanyl) amphotericin B methyl ester DMβAla-AME	$COOCH_3$	$NHCO(CH_2)_2\overset{+}{N}H(CH_3)_2$
12	N-(N'-ethylsuccinimido) amphotericin B NEM-AMB	COO^-	$\overset{+}{N}H_2-CH(CH_2\cdot CO)-N-C_2H_5$
13	N-(N'-ethylsuccinimido) amphotericin B methyl ester NEM-AME	$COOCH_3$	$\overset{+}{N}H_2-CH(CH_2-CO)-N-C_2H_5$
14	N-(N'-ethylsuccinimido) amphotericin B 3-(N', N'-dimethylamino) propyl amide NEM-AMA	$CONH(CH_2)_3\overset{+}{N}H(CH_3)_2$	$\overset{+}{N}H_2-CH(CH_2-CO)-N-C_2H_5$
15	N-(l-deoxy-D-fructos-l-yl)amphotericin B Fru-AMB	COO^-	$\overset{+}{N}H_2-CH_2$ (fructose)
16	N, N, N-trimethylamphotericin B DMS-AMB	COO^-	$\overset{+}{N}(CH_3)_3$
17	N, N, N-trimethylamphotericin B methyl ester DMS-AME	$COOCH_3$	$\overset{+}{N}(CH_3)_3$

Fig. 1. Chemical structure of amphotericin B and its derivatives studied.

B derivatives used were obtained according to the procedures described in reference [6]. The compounds to be tested were dissolved in dimethyl formamide at a concentration of 1 or 10 mg/ml and were applied to the cell suspension in microliter amounts. The concentration of dimethyl formamide did not exceed 2% and this amount did not affect the cells. Solutions of antibiotics were prepared immediately before use. The concentrations were expressed as μg/ml, calculated for pure compounds.

Erythrocytes. Human blood, citrate anticoagulated was kept at 4°. No differences in the sensitivity of cells to polyene macrolide antibiotics were observed when 14-day-old blood was used for cell preparation. Just before use, erythrocytes were separated from plasma and buffy coat by centrifugation for 15 min at 2000 *g*, and then washed three times by suspending in 4 vol. of choline medium, followed by centrifugation. Packed cells were resuspended in 10 vol. of 150 mM choline chloride, pH 7.4 buffered with 5 mM Tris–Cl (stock cell suspension). For antibiotic treatment the stock cell suspension was diluted 25-fold with tested medium. The final cell concentration was *ca.* 2×10^7 cells/ml. After complete lysis and centrifugation the supernatant of this suspension had an absorbance 0.7 at 540 nm.

Determination of haemolytic activity (H_{50}) [7]. Portions (2.5 ml) of cell suspension in 150 mM potassium chloride, pH 7.4, buffered with 5 mM Tris–Cl, were incubated for 15 min at 37° in a shaking water bath, then increasing amounts of the compounds to be tested were added. After 1 hr at 37°, the samples were spun and the absorbance of the supernatant was determined at 540 nm in a spectral colorimeter (Carl Zeiss, Jena). The dosage which led to 50%

haemolysis (H_{50}) was extrapolated graphically from the haemolysis concentration curves obtained from three separate determinations. The value for 100% haemolysis was obtained by hypotonic haemolysis in water.

Determination of potassium release from erythrocytes (EK_{50}). Portions (2.5 ml) of cell suspension in 150 mM choline chloride, pH 7.4, buffered with 5 mM Tris–Cl, were incubated at 37°, in a shaking water bath with increasing amounts of the tested compounds. After 1 hr the samples were spun and the supernatant potassium concentration was determined by flame photometry. The value for 100% of potassium release was obtained for lysed cells.

Determination of antifungal activity (IC_{50}) [8]. The antifungal activity was measured as the concentration of antibiotic causing 50% of growth inhibition (IC_{50}) on *Saccharomyces cerevisiae* (ATCC 9763) and *Candida albicans*. The IC_{50} was determined by the serial dilution method in liquid medium [1% bactopeptone (Difco) and 2% glucose in 0.5% NaCl] inoculated to an optical density of 0.02 at 660 nm ($\sim 10^5$ cells/ml). The extent of growth was monitored turbidimetrically with a colorimeter at 660 nm after 24 hr of incubation at 28°.

The IC_{50} values were determined graphically from dose–response curves.

Determination of potassium release from yeasts (YK_{50}). *S. cerevisiae* cells were isolated from overnight culture growing in liquid medium, composed of yeast extract (0.3%), bactopeptone (0.5%) and glucose (1%). After centrifugation cells were washed twice with distilled water and suspended in 50 mM Tris–Cl, pH 7.4. This suspension was incubated with the desired concentration of an antibiotic at 28° for 1 hr. Then the suspension was centrifuged and the supernatant potassium concentration was determined by flame photometry. One hundred percent of potassium release was obtained for boiled cell suspension.

Preparation of large unilamellar vesicles. Vesicles were prepared according to the method of Szoka and Papahadjopoulos [9] using 40 μmoles of lipid mixture per ml of 100 mM sodium phosphate: 100 mM sodium sulfate solution (pH 5.5). The lipidic composition of negatively charged vesicles was L-α-phosphatidyl choline:phosphatidic acid:sterol (cholesterol or ergosterol) in a molar ratio of 80:10:10. After vesicle formation by reverse phase evaporation, the vesicle suspension was filtered through polycarbonate porous membranes (Nucleopore Corporation, Pleasanton, CA) first using 1 μm and then 0.4 μm pore size.

Spectroscopic measurements. Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrometer. Circular dichroism spectra were recorded with Jobin–Yvon Mark III dichrograph equipped with a Nicolet 1171 signal averager. Studied compounds were added as their Me_2SO solutions in microliter amounts either to vesicles suspension (lipid concentration 1.5×10^{-3} M) or to pure buffer. Final concentrations of antibiotics were 2×10^{-6} M.

Principal component analysis. The five biological activities (Table 1) define a five-dimensional space in which every AMB derivative may be represented as a point.

The biological activities are correlated as shown in Table 2. The dimensionality needed to describe the space of the tested AMB derivatives should be lower than 5 because of the existence of correlation between activities.

Principal component analysis [4, 5] uses linear

Table 1. Activity data

No.	Compound	Yeasts			Erythrocytes	
		IC^*_{50} (μg/ml)	YK_{50} (μg/ml)	IC^*_{50} (μg/ml)	H_{50} (μg/ml)	EK_{50} (μg/ml)
		Saccharomyces cerevisiae		*Candida* albicans		
1	AMB	0.05	0.58	0.03	1.7	0.48
2	AME	0.07	0.28	0.05	4.8	0.76
3	AMA	0.08	0.38	0.04	6.5	1.50
4	Ac–AMB	0.35	8.10	0.28	25.0	9.20
5	Ac–AME	0.55	10.00	0.25	115.0	12.00
6	Ac–AMA	0.25	1.10	0.30	14.0	9.00
7	Gly–AME	0.07	0.31	0.06	4.6	1.40
8	DMGly–AMB	0.08	0.69	0.08	11.0	2.10
9	DMGly–AME	0.12	0.38	0.10	3.9	1.40
10	DMGly–AMA	0.13	0.31	0.12	15.0	8.20
11	DM-β-Ala–AME	0.10	0.22	0.12	2.9	1.10
12	NEM–AMB	0.20	6.30	0.10	100.0	7.20
13	NEM–AME†	>1.00	20.00	>1.00	>200.0	40.00
14	NEM–AMA	0.20	5.60	0.20	7.5	1.10
15	Fru–AMB	0.05	0.50	0.06	3.5	1.70
16	DMS–AB	0.08	1.50	0.06	6.5	1.50
17	DMS–AME	0.07	1.00	0.06	8.0	2.70

Values express concentrations of antibiotics causing: 50% of microbial growth inhibition (IC_{50}); 50% of intracellular potassium release from yeasts (YK_{50}); or erythrocytes (EK_{50}); 50% of haemoglobin release (H_{50}).

* Taken from Ref. [6] for purpose of principal component analysis.

† This compound was not included in principal component analysis.

Table 2. Correlation matrix

	IC_{50} (S.c.)	YK_{50}	IC_{50} (C.a.)	H_{50}	EK_{50}
IC_{50} (S.c.)	1.0000				
YK_{50}	0.8839	1.0000			
IC_{50} (C.a.)	0.8295	0.6546	1.0000		
H_{50}	0.7534	0.7854	0.3795	1.0000	
EK_{50}	0.8449	0.6759	0.7611	0.7124	1.0000

S.c.—*Saccharomyces cerevisiae*, C.a.—*Candida albicans*, remaining symbols are defined in Table 1.

combinations of the activities to replace the starting set of n variables (here $n = 5$) by another set of p orthogonal principal components ($p \leq n$). Every principal component is a linear combination of the starting activities, and the new set is built in such a way that each principal component bears in turn the maximum of variance. The first principal component is directed along the principal axis of the ellipsoid defined by the cloud of points (compounds in the five-dimensional space initially defined). The other principal components are then built from the first one by applying the orthogonality condition within the n-dimensional space.

The process of orthogonalization aims to replace the starting set of more or less correlated variables by a new set of fully independent variables.

Furthermore, there is a quantified hierarchy in the information content of the variable space: the first principal component plays a determining role in the description of the whole population of compounds; the last one plays the least significant role; suppression of the last significant principal component may cause no important distortion in the description of compounds. The distortion incurred by component suppression may be expressed as a percentage of information lost. As a consequence, it is possible to pass from a n-dimensional space to a simpler p-space by the successive removal of n–p components, and at each removal the loss of information is quantitatively assessed. In this work (Table 3) the suppression of two last significant principal components providing an easily visualized three-dimensional space caused a loss of only 2% of the total information. The suppression of three principal components results in an 8% loss of information.

RESULTS

Yeasts: growth inhibition and potassium release

The activity of amphotericin B and its derivatives against yeasts was expressed as antibiotic concentration causing 50% growth inhibition (IC_{50}) and concentration causing 50% of intracellular potassium release (YK_{50}) (Table 1). Extensive analysis of the relationship between the structure of amphotericin B derivatives and growth inhibition has been described in Ref. 6. The most important results of this analysis are:

(i) the sensitivity of *S. cerevisiae* and *C. albicans* to the compounds studied is very similar, therefore *S. cerevisiae* can be conveniently used instead of the pathogenic *Candida albicans* in the other studies;

(ii) chemical modification of amphotericin B carboxyl group does not change the antimicrobial activity;

(iii) presence of the positively-charged nitrogen atom bound to sugar moiety is indispensable for the activity.

YK_{50} values were determined from the dose–response curves, which, for some derivatives, are shown in Fig. 2. Comparison of YK_{50} and IC_{50} (Table 1) indicates the same structural requirements concerning modifications of the amino group for both activities. In contrast to that, YK_{50} data permit us

Table 3. Principal component analysis and loadings of each variable for each factor

(a) Principal component analysis					
Factor	I	II	III	IV	V
Eigen-values	3.9296	0.6517	0.3072	0.0655	0.0460
Explained variance (%)	79	92	98	99	100
Gain of information (%)	13	6	1	1	
(b) Loadings of each variable for each factor					
IC_{50} (S.c.)	0.9761	−0.0628	−0.0840	0.0883	−0.1685
YK_{50}	0.9054	0.1835	−0.3535	−0.1433	0.0323
IC_{50} (C.a.)	0.8193	−0.5541	−0.0644	0.0814	0.1047
H_{50}	0.8194	0.5437	0.1189	0.1158	0.0739
EK_{50}	0.9025	−0.1068	0.3982	−0.1309	−0.0122

Symbols are defined in Table 1.

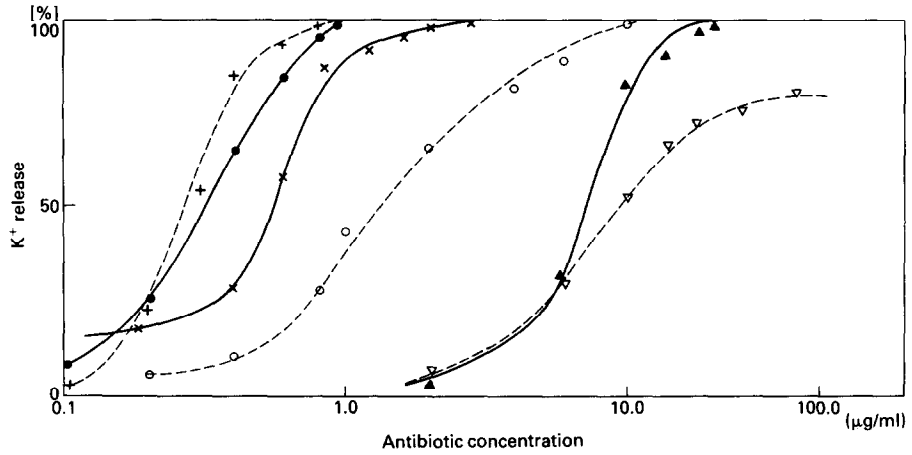

Fig. 2. Dose–response curves obtained for potassium release from *Saccharomyces cerevisiae* for: ×——×, AMB; +--+, AME; ○——○, AMA; ●--●, DMS-AME; △——△, NAc-AMB; ▽——▽, Ac-AME.

to conclude that substitution at the carboxyl group increases permeabilizing activity in yeasts and gives compounds which are more active than amphotericin B (Figs. 2 and 3a). The kind of the substituent seems to be less important for the activity, because methyl esters as well as derivatives obtained by amidation of the carboxyl group caused similar increase of activity. Arrangement of the compounds according to increasing YK_{50} values allows for clear-cut distinction between groups of active and non-active derivatives (Fig. 3a). In long-lasting growth inhibition tests such differences were not detected.

Erythrocytes: permeabilizing and haemolytic activity

In Fig. 4 are shown the dose–response curves of potassium release (a) and haemolysis (b) for amphotericin B and some derivatives.

Unlike the results on yeasts, amphotericin B was the most active compound in both tests (Table 1). For a given compound both curves have a similar S-shape and the haemolytic curves were always shifted to higher concentrations. However, the ratio H_{50}/EK_{50} is 3.5 for amphotericin B and is varied in the range 1.5–14 for the other compounds (Table 1). Haemolytic activity was determined in isoosmotic potassium chloride and the ratios reflect the difference between KCl influx and efflux [7, 10]. No relationship between the ratios and amphotericin B derivatives structure was detected.

The arrangement of the compounds according to the decreasing permeabilizing activity (Fig. 3b) allows us to distinguish two groups. The first group contains active derivatives; all these compounds have positively-charged nitrogen atom bound directly or

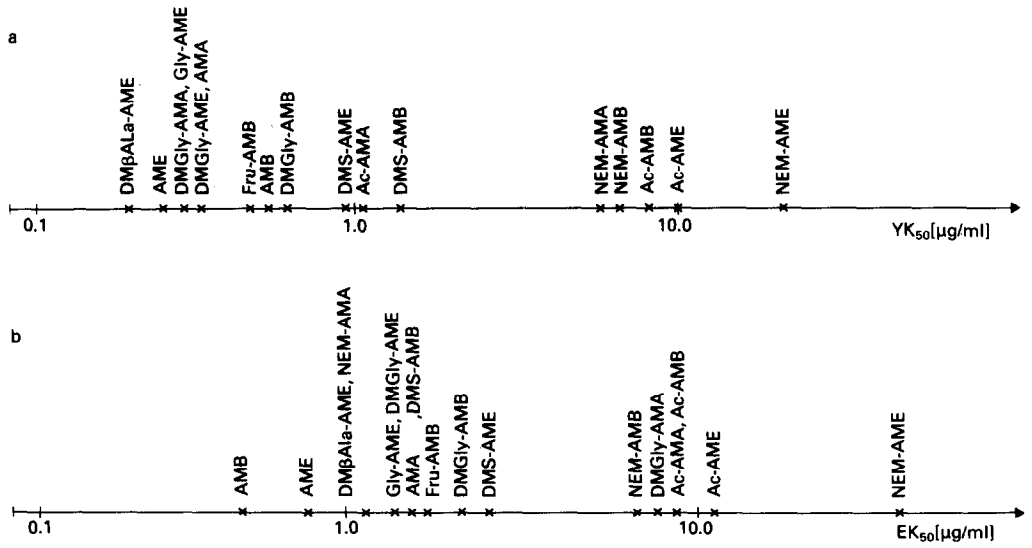

Fig. 3. Comparison of potassium release activity on yeasts (a) and human red blood cells (b). Activity is expressed as the concentration of compound causing 50% release of intracellular potassium after 1 hr incubation.

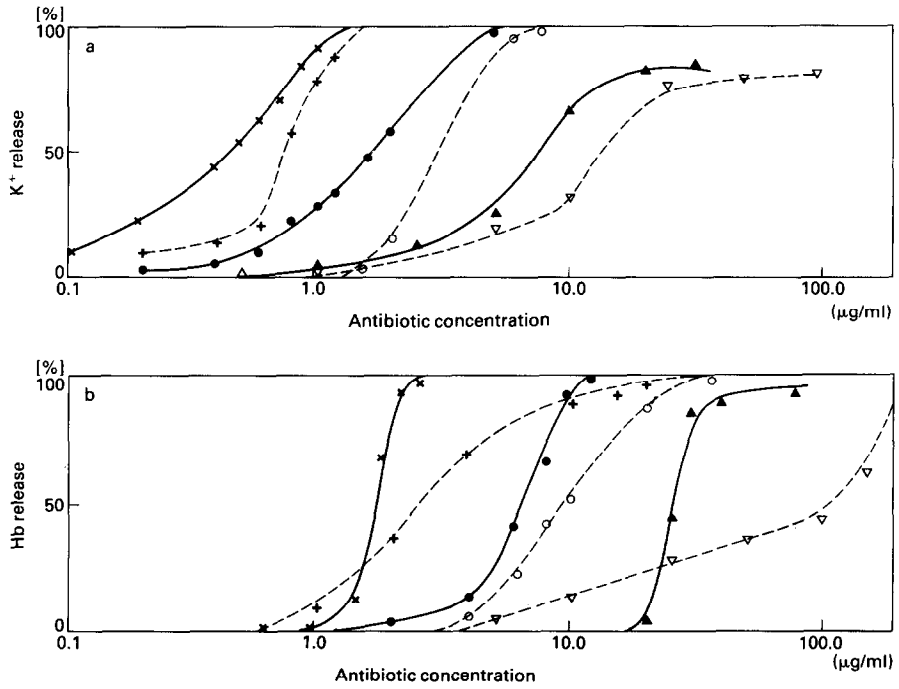

Fig. 4. Dose–response curves obtained for erythrocytes: (a) potassium release and (b) haemolysis for: ×——×, AMB; +--+, AME; ○——○, AMA; ●---●, DMS-AME; ▲——▲, Ac-AMB; ▽——▽, Ac-AME.

indirectly to sugar moiety. The relation between substitution of the carboxyl group and activity is inexplicit. The second group comprises low active compounds, i.e. *N*-acetyl and NEM derivatives as well as their methyl esters. All these compounds were also poorly active on yeasts.

Low permeabilizing activity was exhibited also by the AMA and DMGly–AMA, compounds relatively active on yeasts.

Relationships between chemical structures and biological properties

For the determination of the relationships between chemical structure of amphotericin B derivatives and biological properties the principal component analysis has been applied. For this numerical analysis the original data set presented in Table 1 was standardized in the following way: from each original value the mean value of the test was subtracted and the result was divided by standard deviation of all results of that test.

In Table 2 the correlation matrix between tests is presented. Correlation coefficients between tests are all positive and rather high: it suggests that activity in all tests used depends mainly on the same chemical features.

The principal component analysis based on this correlation matrix shows (Table 3) that three first components explain practically all the activity variance (taking into consideration the experimental errors of the data set).

In the first component, the main factor which explains 79% of activity variance, all biological tests have positive and high loadings. For that reason the first principal component may be interpreted as a measure of "general biological activity".

Figure 5 shows the vector representation of the loadings of tested activities in the plane of second and third principal component. In the second principal component antifungal activity, IC_{50} (*C. albicans*), participates with high negative loading, but haemolytic activity (H_{50}) with a high positive one. Thus, the second component may be a measure of ergosterol vs cholesterol selectivity based on antifungal activity vs haemolytic activity (IC_{50} vs H_{50} selectivity).

In the third component, the activities determined on ergosterol-containing cells (IC_{50} and YK_{50}) have opposite sign of loadings as compared to the activities determined in cholesterol-containing cells (H_{50} and EK_{50}). The highest absolute values of loadings in this component are for potassium leakage tests, but with a negative sign for yeasts and a positive one for erythrocytes. Thus, the third principal component may also be a measure of ergosterol vs cholesterol selectivity based on activities measured by potassium release (YK_{50} vs EK_{50} selectivity).

The relationships between the chemical structure of amphotericin B derivatives and their biological features, represented by three main components, are shown in Fig. 6.

The following results have been obtained:

1. *General biological activity* (Fig. 6a). *N*-acetylation as well as formation of NEM derivatives of AMB, AME and AMA decrease activity. This effect is particularly strong in the case of AME derivatives, but is weaker in the case of AMA ones.

Alkylation or aminoacylation of amino group of amino–sugar moiety as well as formation of ester or

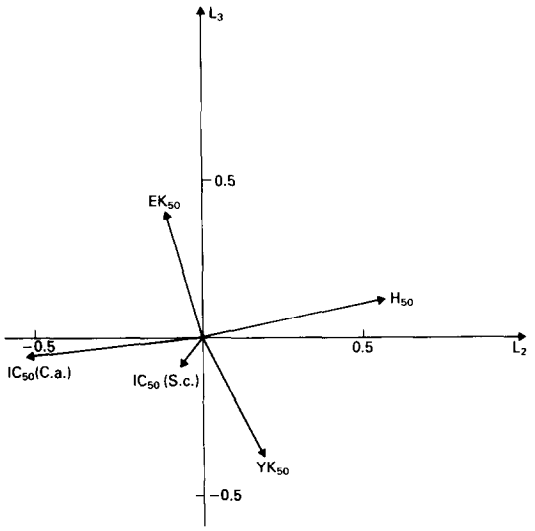

Fig. 5. The vector representation of the biological variables loadings in the plane of second (L_2) and third (L_3) principal components.

amide on carboxyl group does not change significantly the activity.

2. *Ergosterol vs cholesterol selectivity*

(a) *Antifungal vs haemolytic selectivity* (Fig. 6b).

Particularly high values of this component are exhibited by Ac–AME and NEM–AMB. However, both compounds have very low antifungal activity and for that reason they cannot be taken into consideration as potential antifungal drugs.

Large group of compounds: AME, AMA, aminoacyl-, DMS- and fructosyl derivatives exhibit better than AMB selectivity. It suggests that substitution of carboxyl group as well as alkylation and/or shift of ionizable amino group improve antifungal vs haemolytic selectivity.

(b) *Ergosterol vs cholesterol permeabilizing selectivity*

Among tested compounds with high activity only DMGly–AMA exhibits this selectivity significantly better than AMB. But only one aminoacyl derivative of AMA has been tested and that observation cannot be generalized to all aminoacyl derivatives of AMA.

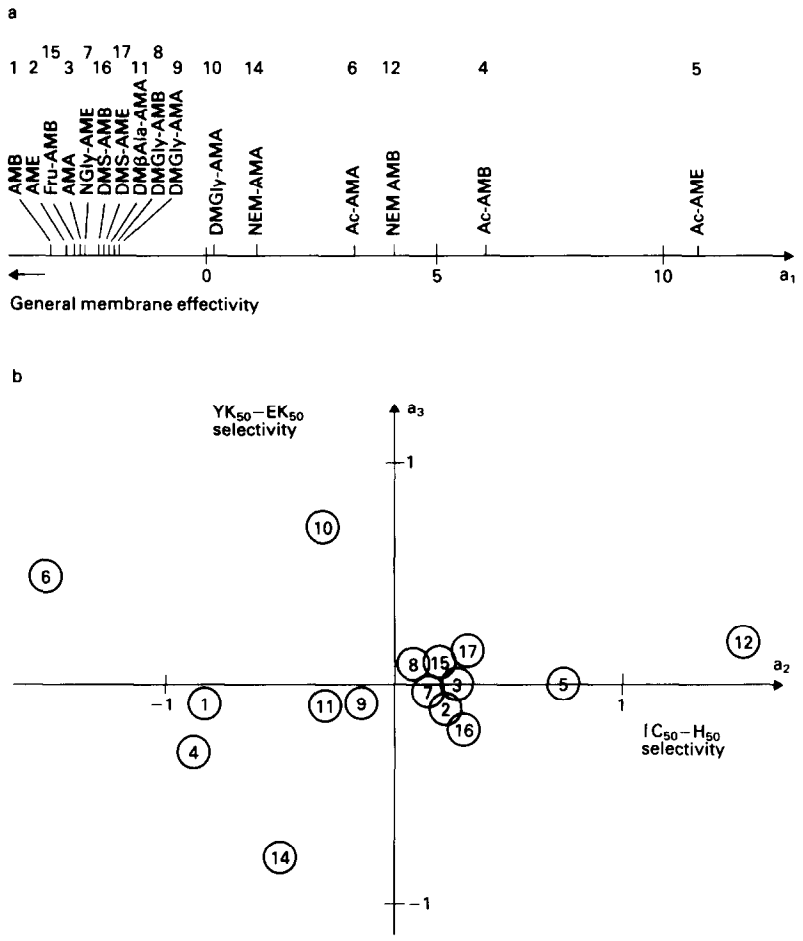

Fig. 6. Representation of the tested compounds in the space of principal components (a) projection on the axis of first principal component; (b) projection on the plane of second and third principal components.

Spectroscopic measurements

The characteristic optical properties of the polyene antibiotics, due to the presence of a system of conjugated double bonds in these molecules, make the study of their interactions with lipids by spectroscopic methods particularly attractive [11–15].

In this study the absorption and circular dichroism spectroscopy have been applied. Measurements were done on large unilamellar vesicles (LUV), which are better models of cells than the small ones [14]. With cells, due to light scattering interference, measurements were not done. Amphotericin B derivatives of different biological activity and ergosterol/cholesterol selectivity have been chosen. Results were referred to spectra of amphotericin B which were taken in the same experimental conditions.

Absorption spectroscopy. It has been shown [11] that in aqueous solutions absorption spectra of amphotericin B are concentration-dependent. Below 5×10^{-7} M the spectrum is characterized by three strong absorption bands at 410, 384 and 365 nm and is assigned to the monomeric form of the antibiotic. Above this concentration, the spectrum progressively changes and at concentrations higher than 10^{-4} M a new spectrum is observed. As compared to the spectrum of monomeric form 410 nm and 384 nm bands are red-shifted and less intense, band 365 nm is not recognizable besides, new, the most intense band at 329 nm appears. This spectrum is attributed to the aggregated form of the antibiotic. In the presence of sterol-containing lipidic vesicles, at high lipid-to-antibiotic ratio another new spectrum is observed. This spectrum has four bands, positions

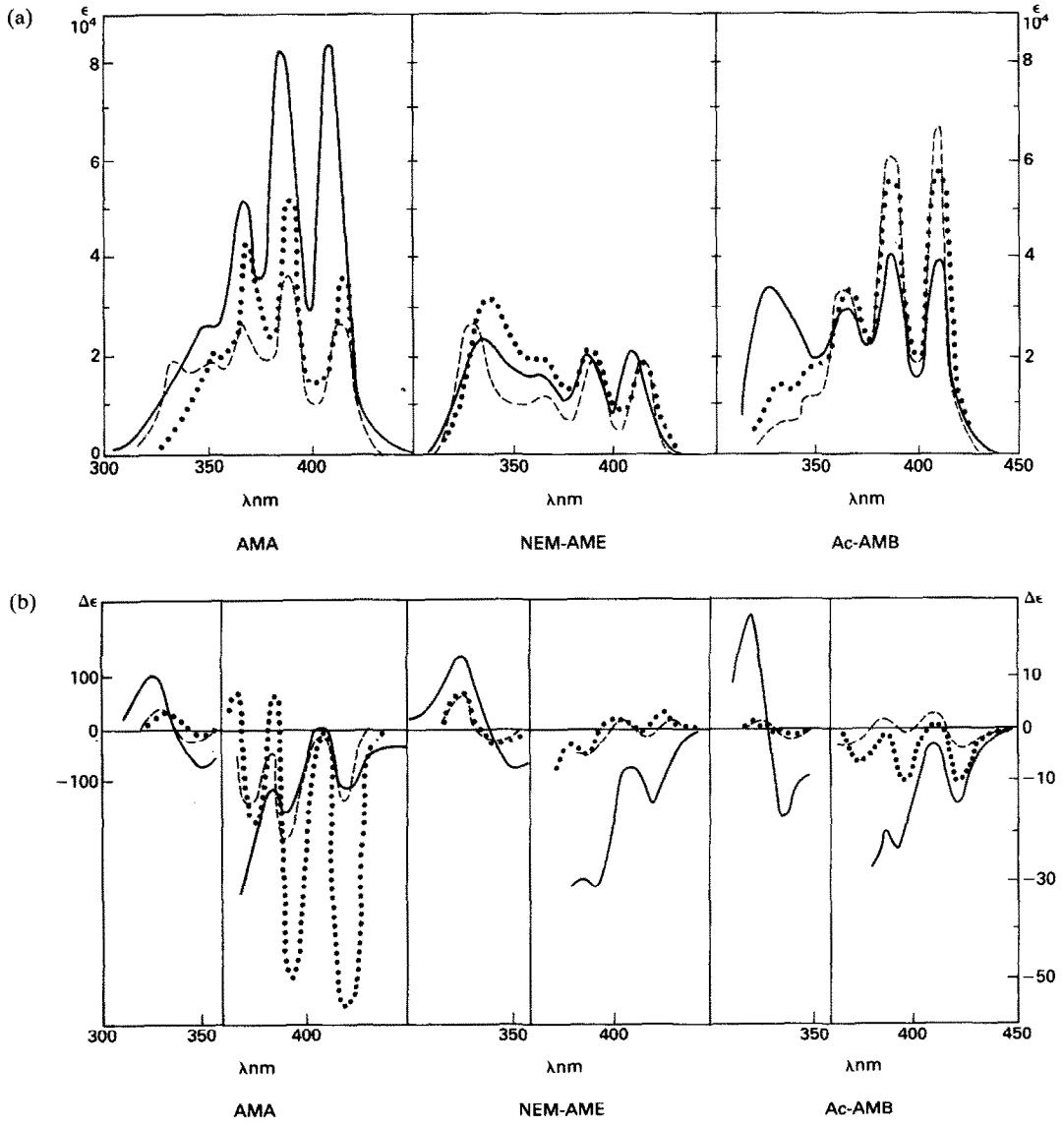

Fig. 7. Absorption (a) and C.D. (b) spectra of some derivatives of AMB: ——, in free medium; – – –, in the presence of cholesterol-containing vesicles; . . . ., in the presence of ergosterol-containing vesicles. Concentration of antibiotics was 2×10^{-6} M. Lipids–antibiotic ratio was around 1000.

Table 4. Values of λ (in nm) for absorption band near 410 nm for AMB and some derivatives

	AMB	AME	AMA	Fru-AMB	NEM-AME	Ac-AMB
Free	410	410	410	410	410	410
Ergosterol vesicles	416	414	414	414	414	410
Cholesterol vesicles	410	414	414	414	414	410

of which are dependent on sterol structure and concentration. Maximal change is usually observed for 410 nm band which in the case of ergosterol is shifted to 416 nm and for cholesterol to 414 nm. In this study, change in the position and intensity of 410 nm band has been taken as a marker of antibiotic–sterol interaction.

Among amphotericin B derivatives studied, in aqueous solution and antibiotic concentration 2×10^{-6} M only AMA exists mainly in the monomeric form. In these conditions AMB and other derivatives (AME, Fru–AMB, NEM–AME, Ac–AMB) are considerably aggregated (Fig. 7a).

Interaction with vesicles was dependent on antibiotic and sterol structures. In the presence of cholesterol-containing vesicles the 410 nm band did not change the position for AMB and Ac–AMB whereas it was shifted to 414 nm for remaining derivatives studied. In the presence of ergosterol-containing vesicles this band was shifted to 416 nm for AMB and to 414 nm for AME, AMA, Fru–AMB, NEM–AME. Its position was not changed in the case of Ac–AMB (Fig. 7a; Table 4). Intensity of all bands was decreased in the case of AMA, AME, Fru–AMB, not changed for NEM–AME and increased for Ac–AMB.

(b) *Circular dichroism* (CD). The monomeric form of AMB exhibits CD spectrum with three weak positive bands at 410, 385 and 365 nm. In CD spectra of the aggregated form of AMB very intense dichroic doublet centered at 329 nm as well as negative bands at 420 and 388 nm are observed. In the presence of sterol-containing lipidic vesicles spectra characterized by strong sharp negative bands between 430 and 380 nm were detected. These types of spectra are assigned to "permeabilizing species", i.e. antibiotic–sterol complex formed in the membrane and responsible for increase membrane permeability [13]. In this work this spectrum is called A. Intensity of the spectrum A is related to polyene sterol affinity. Higher spectrum intensity means stronger complex. Results obtained by CD spectroscopy show that aqueous solutions of all antibiotics studied contain antibiotics in aggregated form. Due to the high sensitivity of the CD method aggregates in solution of AMA also could be detected.

In the presence of vesicles antibiotic–sterol interactions were observed for AMB, AME, Fru–AMB for both sterols tested. However, for all these compounds the intensity of spectrum A is considerably higher for ergosterol-containing vesicles than for cholesterol-containing ones.

In Fig. 7b spectra for AMA are shown as an example. For Ac–AMB only in the case of ergosterol-containing vesicles spectrum A is observed, but has very low intensity. In the case of NEM–AME for none sterol spectrum A is detected.

For all compounds tested dichroic doublet centered at 329 nm, characteristic for the aggregated form of polyene antibiotic, disappeared in the presence of lipidic vesicles (Fig. 7b).

DISCUSSION

Structural requirements of the membrane sterol necessary to confer polyene sensitivity have been established in many studies on cells [16–18], liposomes [19] and black films [20]. The membrane sterol must have a 3-α-hydroxyl group, a planar molecule and hydrophobic side chain at the C-17 position. Cholesterol and ergosterol occurring in mammalian and fungal cells respectively fulfil these requirements. On the other hand, not enough is known about structural requirements of polyene macrolides for the interaction with sterols and especially for cholesterol–ergosterol differentiation. However, some general rules have been proposed. The most important structural features determining the activity and cholesterol vs ergosterol selectivity seem to be the length of the polyenic chromophore and charge of the molecule [2, 8, 21]. It has been suggested that formation of sterol antibiotic complex is due to (i) hydrophobic interaction with sterol ring system and π-electronic interaction of sterol double bond(s) with polyenic chromophore of the antibiotic and (ii) the interaction of sterol OH group with ionizable polar groups of the antibiotic [21, 22].

The purpose of the present study was to analyze the significance of ionizable polar groups (amino and carboxyl) for the biological activity and selective toxicity of amphotericin B. In these studies the properties of the above functional groups were rationally changed by chemical modifications.

The principal component analysis shows that biological activity of amphotericin B derivatives in all tests applied depends primarily on the properties of nitrogen atom(s) present at the polar head of the antibiotic molecule. High biological activity is exhibited by compounds in which the nitrogen atom is positively charged at physiological pH. The location of a nitrogen atom is less important, as similar activity is exhibited by compounds in which this atom is bound directly to sugar residue and by those in which it is shifted to the aminoacyl side chain. The hydrogen atoms bound to the nitrogen are not indispensable and they can be replaced by alkyls.

Drastic decrease of the biological activity was observed for *N*-acetyl derivatives, in which the nitrogen atom can not acquire the charge (*N*-acetyl

AMB). Similar loss of activity was observed for NEM derivatives, but in this case inactivation cannot be explained by the lack of chargeable nitrogen atoms. The explanation of properties of NEM-derivatives is given in the Ref. [6]. The presence of negative charge at carboxyl group is not essential for biological activity. It is interesting to note that the introduction of positively charged nitrogen atoms by appropriate substitution at carboxyl group (e.g. AMA) causes partial recovery of the biological activity of inactive *N*-acetyl and NEM derivatives.

The ergosterol vs cholesterol selectivity was analyzed on the basis of two parameters:

(i) microbial growth inhibition and haemolysis;
and (ii) permeabilizing activities on yeasts and erythrocytes.

The analysis shows that for the active compounds selectivity is related mainly to the substitution at carboxyl group and only to a minor degree to the substitution at the nitrogen atom.

Results obtained by spectroscopic methods showed that biological activity is reflected in the interaction between antibiotic and membrane sterols. Non-active compounds had restricted ability of such an interaction. For derivatives, similarly as for amphotericin B [13], stronger interaction with ergosterol-containing membranes than with cholesterol-containing ones was detected.

The occurrence of the interaction is determined by the properties of the polar groups of the antibiotic. Indispensable for the interaction is the presence of the positively charged nitrogen atom. Lack of negative charge at carboxyl group favours interaction with ergosterol. Molecular mechanisms of these effects are not clear as yet and are under further studies.

In conclusion it can be said that two chemical features are especially important for biological properties of amphotericin B derivatives:

(i) presence of protonable or bearing fixed charge nitrogen atom in amino-sugar moiety or in aminoacyl side chain attached to amino-sugar (condition for biological activity);

(ii) absence of free negatively charged carboxyl group (the condition for ergosterol vs cholesterol selectivity).

Two compounds, AMA and DMS–AME, fulfil the above structural requirements. These compounds appeared to be less toxic and more effective than native amphotericin B in the treatment of systemic candidiasis in mice [23].

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