VOL. 57 NO. 10, OCT. 2004 THE JOURNAL OF ANTIBIOTICS pp. 669 — 678

Studies of the Effects of Antifungal Cationic Derivatives of Amphotericin B on Human Erythrocytes

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(Received for publication August 16, 2004)

The novel group of amphotericin B (AmB) cationic derivatives has been examined in terms of relationship between self-association and selective toxicity. In all determinations AmB has been used as reference compound. *In vitro* toxic effects of the compounds on human erythrocytes were determined by measuring leakage of intracellular potassium ions and hemolysis. Antifungal effects were determined as MIC and intracellular potassium loss. The compounds self-association was followed by UV-Vis spectroscopy. The results suggested that: i) unlike AmB the monomer/self-associated species ratio is not an essential in governing the selective toxicity of the derivatives studied; ii) the presence of a bulky substituent in the AmB molecule, preferably located at the amino group of mycosamine moiety is the structural factor essential for the selective toxicity improvement.

Amphotericin B (AmB), a broad spectrum fungicidal and active on multidrug resistant strains polyene macrolide antibiotic, however highly toxic, still remains the most valuable among antifungal drugs currently used in the treatment of deep-seated disseminated mycotic infections. High toxicity of AmB is manifested in various short- or long- term side effects[1]. Nephrotoxicity and hemolytic activity are main factors limiting its clinical application[1]. Poor selective toxicity of AmB results from the same molecular mechanisms of antifungal action and toxicity towards mammalian host cells[2~5]. In both types of cells AmB acts at the cell membrane level inducing modification of membrane structure leading to the lethal permeability changes. Cell sensitivity to AmB is determined by sterol composition of the plasma membrane. Some differences in the relative affinities of AmB to ergosterol (the main fungal sterol) and cholesterol in mammalian cells have been considered to be major determinant of selective toxicity and therapeutic usefulness of the antibiotic. Interaction of AmB with membrane phospholipids seems to be not essential for the antibiotic action on fungal as well as mammalian cells. Besides, AmB-induced lipid peroxidation and modulation of the activity of ionic pumps and of some other membrane enzymes may also play a role in final toxic effects of the antibiotic[3].

It is well documented that selectivity of AmB is also in part related to the physical state of antibiotic in the medium[6,7]. AmB in aqueous medium undergoes self-association and is present as a mixture of monomers and various soluble and insoluble aggregates in equilibrium. It has been demonstrated that AmB in monomeric form is able to create permeability pathway only in ergosterol-containing membranes whereas soluble aggregates are effective on both: ergosterol- and cholesterol-containing ones[6]. The AmB solubility problem and self-association aspect can be rationally controlled by: a) chemical modification of the antibiotic[8~11], b) surface active agents[12], c) appropriate lipidic formulations of the drug[2,3,13].

Although, liposomal and other lipidic formulations of AmB considered to be a reservoir of its nontoxic monomeric form undoubtedly constitute a progress in developing less toxic drug, this progress is still insufficient for the safe clinical use of this otherwise valuable antifungal antibiotic. In our opinion the hidden high therapeutic potential of AmB, being hindered by high toxicity of native compound, can be exploited in full only by chemical modification of the antibiotic molecule[15].

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The above discussed mechanisms of selective toxicity were established for AmB, which is a very poorly selective compound. In our opinion these mechanisms do not fully explain the action of highly selective AmB derivatives. The discovery of *N*-methyl-*N*-D-fructopyranosyl AmB methyl ester (MFAME)[16] exhibiting dramatically improved selectivity, indicates that other mechanism(s) of selective toxicity should be also taken into account. This compound, practically ineffective on cholesterol containing cells, does not exhibit essential differences in the affinity to both sterols in bimolecular systems[17]. Also other factors considered as essential for the selectivity of AmB action, like cell membrane affinity[18], and extend of self-association[19] appeared to be not crucial for MFAME. We have postulated that high selectivity exhibited by MFAME is due to the particular properties of the complexes formed upon the drug interaction with cholesterol containing membranes that makes them unable to form permeabilizing species[19]. It has been also postulated that this favorable effect is induced by the presence of bulky substituent in the modified molecule what may influence the characteristics of drug-cholesterol interaction[15].

To support our above concept the larger number of AmB derivatives with bulky substituents should be examined.

The subject of this paper is the examination of the effects of a novel group of antifungal cationic AmB derivatives on human erythrocytes. These compounds have been developed as nonviral, effective vectors for antisense strategy and gene therapy[20]. Their ionic complexes formed with ODN's efficiently protect the oligonucleotides against blood serum nucleases, and induce their internalization into mammalian cells with excellent yield[21~24].

The examined compounds are listed in Table 1. Their cationic character results from appropriate substitution of the "polar head" of the antibiotic at its amino and carboxyl groups. As compounds of basic character they form well water soluble salts. The presence of a net charge in their molecule should hinder their self-association and prevent formation of insoluble aggregates[8] what allows to examine the role of the state of the compounds in aqueous media in their selective toxicity in the wider concentration range. The derivatives are also characterized by the presence of bulky groups.

In the present studies the effect of the above structural changes in AmB molecule on self-association and selectivity towards fungal and mammalian cells has been examined. UV-Vis spectroscopy has been applied to estimate the state of compounds in aqueous media. Their antifungal activity was determined on *Candida albicans*. Toxicity for mammalian cells has been determined on human erythrocytes. Native AmB has been used as reference compound in all determinations.

Materials and Methods

AmB and Its Derivatives

AmB was Sigma-Aldrich product. All derivatives studied were synthesized in the Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology. Amphotericin B 3-dimethylaminopropyl amide (AMA) was obtained according to the procedure described[25]. Amphotericin B 4-methylpiperazine amide (AMPA) and amphotericin B 2-(4-morpholyl)ethyl amide (AMEA) were obtained from AmB and corresponding amines using DPPA method as described[20,25]. Amphotericin B 4-methylpiperazine hydrazide (HAMA) was prepared according to the method described[26]. *N*-(*N*′-3-Dimethylaminopropylsuccinimido) amphotericin B methyl ester (SAME) was obtained by methylation of *N*-(*N*′-3-dimethylaminopropylsuccinimido) amphotericin B with diazomethane[20,27]. *N*,*N*,*N*-Trimethylamphotericin B methyl ester chloride (DMS-AME) was prepared as it was described[28]. *N*-D-Ornithylamphotericin B 3-dimethyl-aminopropyl amide (Orn-AMA) was obtained in the reaction of AMA with N^{α}>,N^{δ}-difluorenemethoxycarbonyl-D-ornithine by DPPA method followed by Fmoc-deprotection using diazobicyclononene (DBN)[20]. *N*-D-Ornithylamphotericin B methyl ester (Orn-AME) was prepared according to WRIGHT *et al.*[29]. Structures of the compounds are presented in Table 1.

Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) usually at concentration 1 mg per ml for AmB and 20 mg per ml for derivatives. Purity of the compounds was determined by electronic absorption of their methanolic solutions ($\varepsilon_{408}=160000\,\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$).

Cells

Candida albicans ATCC 10261 was maintained on agar slants composed of bacto-peptone (Difco) 1%, yeast extract (Difco) 1%, glucose (Polfa) 2% and agar (Difco) 2% and multiplied in the medium in which agar was omitted. Growth temperature was 30°C.

Erythrocytes: Human blood from a local blood bank citrate anticoagulated was kept at 4°C. Just before use, erythrocytes were separated from plasma and buffy coat by centrifugation (10 minutes, 3000×*g*, 4°C) and then washed three times by suspending in saline, followed by centrifugation.

Determination of Antifungal Activity

Antibiotics minimum inhibitory concentration (MIC) was determined on *Candida albicans* ATCC 10261 by the serial dilution method in liquid medium containing 2% glucose and 1% bacto-peptone. For MIC, determination cell suspension containing 10^5 cells per ml ($A_{660}\approx0.01$), prepared from 24 hours culture, was divided into 2 ml portions. Solutions of AmB and derivatives were prepared in DMSO at concentration 100-fold higher than desired final one just before use. 20 μl of the proper antibiotic solution was added to 2 ml cell suspension samples. The lowest concentration of the antibiotic yielding no growth after 24 hours of incubation at 30°C was defined as MIC. Samples were prepared in duplicates and determination was repeated.

Determination of Potassium Release from *Candida albicans* (t_{50})

Cells from 24 hours culture were harvested by centrifugation (10 minutes, 3000×*g*), washed with saline and resuspended in saline at concentration 2×10^8 cells per ml. Then potassium ion selelectrode (F2312K, Radiometer, Copenhagen) was introduced to the cell suspension. When the recorded signal had stabilized, the antibiotic solution was added and potassium level in suspension was continuously monitored during 30 minutes. The concentration of potassium in the medium was determined according to a calibration curve obtained for various concentrations of potassium chloride in saline. 100% of potassium release was obtained for boiled cell suspension. Error of the determination was less than 5%.

Determination of Potassium Release from Erythrocytes and Their Hemolysis

The solutions of tested compounds in DMSO were prepared at concentration 100-fold higher than required final one. 30 μl of antibiotic solution was added to 3 ml samples of saline and then the proper amount of the dense erythrocyte suspension was added to each sample; final erythrocytes concentration was 10^8 cells/ml. Samples were incubated for 30 minutes at 30°C with shaking. The amount of potassium released into the medium was measured with potassium ion selective electrode and its concentration was determined according to a calibration curve. Moreover, degree of hemolysis was determined in each sample. For this purpose the samples were centrifuged (4 minutes, 3000×*g*) and absorbance of each supernatant, diluted 10-fold with saline, was measured at 540 nm. The values of 100% hemolysis and potassium release were obtained in sample hemolysed in water. In each experiment samples were prepared in duplicates and experiments were repeated. Error of the determination was less than 5%.

Self-association of AmB and Its Derivatives

Self-association of the compounds studied was monitored by UV-Vis spectroscopy. Serial dilution of the compounds were prepared in DMSO at concentration 100-fold higher than desired final one. Then 20 μl aliquot of each dilution were added to 2 ml of methanol or to 2 ml of saline. Samples in saline were incubated for 30 minutes at 30°C. UV-Vis spectrum of each sample was recorded at the range of 300~430 nm (Beckman, model 3600 spectrophotometer) using 1.0 or 0.1 cm path length quartz cells. The ratio of absorbance at 348 and 409 nm, $s=A_{348}/A_{409}$, was used to estimate the degree of the antibiotic self-association[7].

Results

Characterization of the Derivatives

The AmB derivatives studied in this work (Table 1) were obtained by chemical modification of the ionizable polar groups of the parent antibiotic, comprising the amino group of the mycosamine moiety and C-16 carboxyl. The amino group was modified by alkylation or aminoacylation, whereas the carboxyl group was modified by amidation or esterification. In case of amides, carboxyl group was substituted with aliphatic (AMA, Orn-AMA) or heterocyclic ring (AMPA, APEA, AMEA, HAMA) residues. Besides Orn-AMA, amino group at mycosamine moiety was free in all other amides. In case of *N*-alkyl and *N*-aminoacyl methyl esters, amino group of aminosugar was also substituted and contained heterocyclic (SAME), aliphatic (DMS-AME) or aminoacyl (Orn-AMA) residues. At physiological pH all derivatives are positively charged whereas parent AmB is zwitterionic.

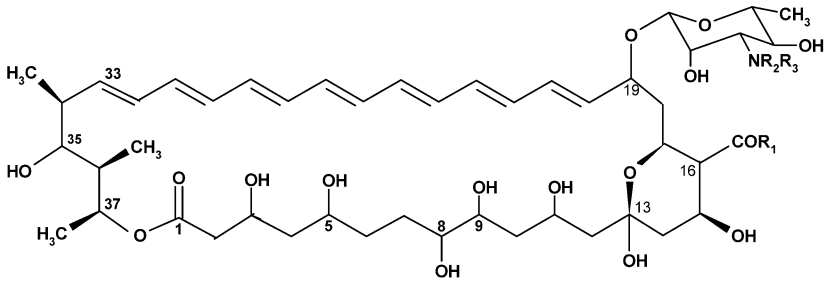
Spectroscopic Properties and Self-association

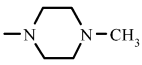
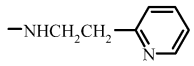
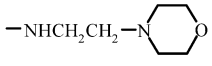
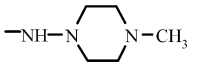
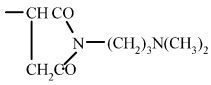
The self-association of AmB and derivatives (Table 1) in saline has been followed by measurement of the electronic absorption in the UV-Vis region as a function of compounds concentration.

The absorption spectra of AmB and selected derivatives: AMA, Orn-AMA and Orn-AME in methanol are shown in Fig. 1 as an example.

The spectroscopic properties of AmB as well as its derivatives substituted at the ionizable polar groups are determined by the presence of the all-*trans* heptaenic

Table 1. Structures of AmB and derivatives.

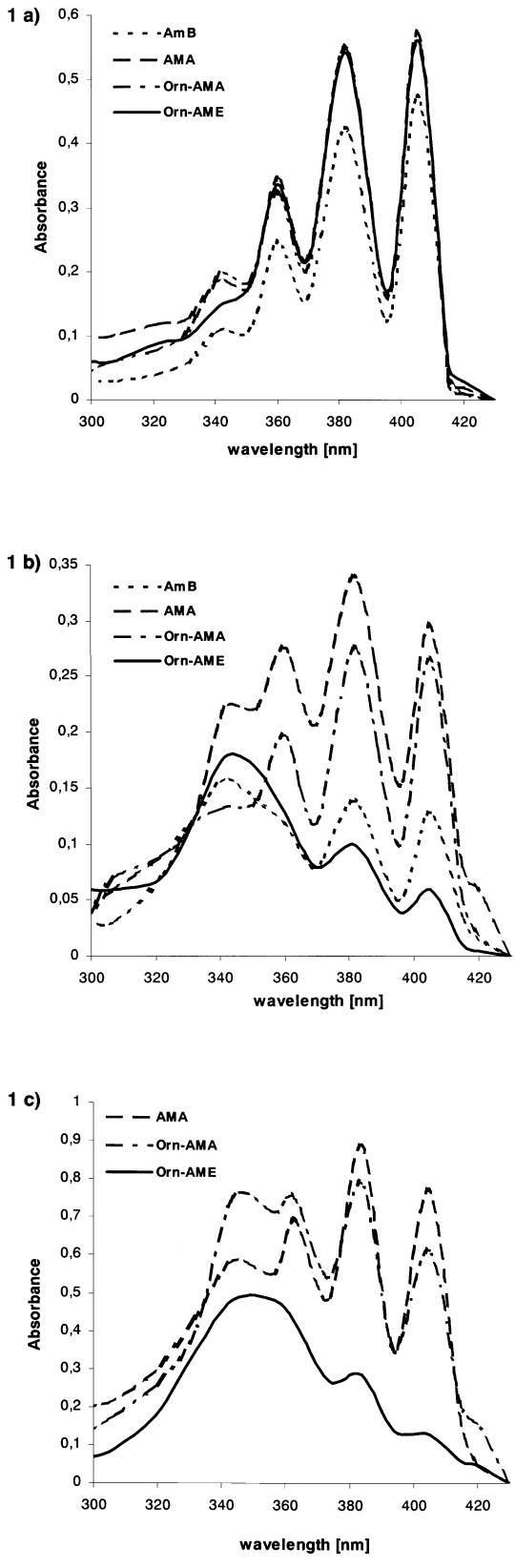


Group	No	Compound	R_1	R_2	R_3	M[g/mol]
Reference	1	AmB	-OH	-H	-H	923
Amides	2	AMA*	$-NH(CH_2)_3N(CH_3)_2$	-H	-H	1006
	3	AMPA		-H	-H	1005
	4	APEA		-H	-H	1027
	5	AMEA		-H	-H	1035
	6	HAMA		-H	-H	1020
***N* – alkyl methyl esters**	7	SAME	$-OCH_3$		-H	1119
	8	DMS-AME**	$-OCH_3$	$(CH_3)_2$	$-CH_3$	980
***N* - aminoacyl methyl esters**	9	Orn-AME	$-OCH_3$	$H_2N(CH_2)_3CH(NH_2)CO-$	-H	1051
***N* - aminoacyl amides**	10	Orn-AMA	$-NH(CH_2)_3N(CH_3)_2$	$H_2N(CH_2)_3CH(NH_2)CO-$	-H	1137

* AMA was used as L-aspartate salt; M=1273 [g/mol].
** DMS-AME was used in the form of chloride; M=1015.5 [g/mol].

chromophore. In polar organic solvents like methanol AmB exists as a monomer and its UV-Vis spectrum is characterized by sharp bands at 408, 383, 364 and 346 nm (Fig. 1a). Absorption spectra of the methanolic solutions of the derivatives listed in Table 1 are similar to the spectrum of the monomeric form of the parent antibiotic. No shifts of typical bands are observed, the bands proportions are not concentration dependent and bands intensities obey the Lambert-Beer's law up to concentration 10^{-4} M. On the contrary, the spectra of aqueous solutions of derivatives, like spectra of AmB, are concentration dependent. Their shapes suggest coexistence of more than two different spectral species. The characteristic changes in the UV-Vis spectra of AmB reflecting its self-association are: disappearance of the band typical for the monomeric form at 409 nm and appearance of new ones. The most intensive is a wide band at 348 nm. The ratio of absorbances A_{348}/A_{409} (s) has been applied as a measure of the degree of AmB self-association[7]. For monomeric AmB the ratio is about 0.25 while for the aggregated forms increases with concentration and saturates at a level about 2. Spectra of the derivatives in saline are subjected to similar concentration dependent changes indicating self-association. However, changes in the overall shape of absorption spectra and

Fig. 1. Absorption spectra of 3 μM AmB; 3.5 μM AMA; 3.5 μM Orn-AME and 3.7 μM Orn-AMA in a) methanol b) saline and c) 16 μM AMA, Orn-AME and 17 μM Orn-AMA in saline; (1 cm path length).



intensities of the bands at 409 and 348 nm are dependent on derivative structures. For example (Fig. 1b), at similar antibiotic concentrations (3 μM) absorption at 409 nm and spectrum shape show that AMA and Orn-AMA are less aggregated whereas Orn-AME is more aggregated than the parent antibiotic. At higher concentrations, in which AmB forms insoluble aggregates, in solutions of AMA and Orn-AMA proportions of the monomeric forms are still high and insoluble aggregates are not formed (Fig. 1c). Assuming that the absorbance ratio s, reflects the extent of the antibiotic aggregation, c_s-concentration of antibiotics at $s=1$ were determined (Table 3). At these concentrations, proportions of monomeric and aggregated forms for all tested compounds should be similar. According to this criterion SAME, DMS-AME and Orn-AME exhibit the same tendency to self-association as AmB. For other derivatives the same degree of self-association occur at much higher concentrations. For AMA $s=1$ was obtained at concentration 25-fold higher than for AmB, whereas for remaining derivatives at about 10-fold higher. However, c_s values indicate that there is no clear correlation between the type of modification and tendency to self-association. In the group of amides, derivatives containing bulky, heterocyclic ring would associate easier than AMA, containing aliphatic substituent. On the contrary, *N*-alkyl methyl ester SAME with voluminous substituent at the amino group has been less disposed to associate than DMS-AME. In aminoacyl derivatives, ester, Orn-AME, would associate easier than amide, Orn-AMA.

Antifungal Activity and Red Blood Cells Toxicity

Candida albicans and human erythrocytes had been chosen as the representatives of pathogenic and host cells, respectively. Biological properties of the derivatives and parent AmB are presented in Tables 2 and 3.

A measure of antifungal activity was a minimal concentration of the drug (MIC) required for complete *Candida albicans* growth inhibition over fixed period of time. MIC values (long-term activity) indicate that antifungal activity of the parent AmB is well preserved in the derivatives studied. Among tested compounds AMA and AMEA showed the same activity as AmB and relatively small (2~4 fold) decrease of activity was observed for the remaining derivatives. Action of AmB and derivatives on *Candida albicans* was also compared by induction of the intracellular potassium efflux (short-term experiment). The results obtained (Table 2) indicate that derivatives like AmB induce potassium efflux. However, besides AMA time required for initiation of potassium

Table 2. The effect of examined compounds on *Candida albicans* ATCC 10261.

Group	No	Compound	MIC [μM]	t_{50} [min]	c[μM]
Reference	1	AmB	0.1	11.5	10.8
Amides	2	AMA	0.08	9	8
	3	AMPA	0.25	20	10
	4	APEA	0.24	23	9.7
	5	AMEA	0.14	25	9.7
	6	HAMA	0.39	16	9.8
***N* – alkyl methyl esters**	7	SAME	0.27	21	8.9
	8	DMS-AME	0.39	18	10.2
***N* – aminoacyl amides**	9	Orn-AMA	0.35	34	8.5
***N* – aminoacyl methyl esters**	10	Orn-AME	0.38	50	9.5

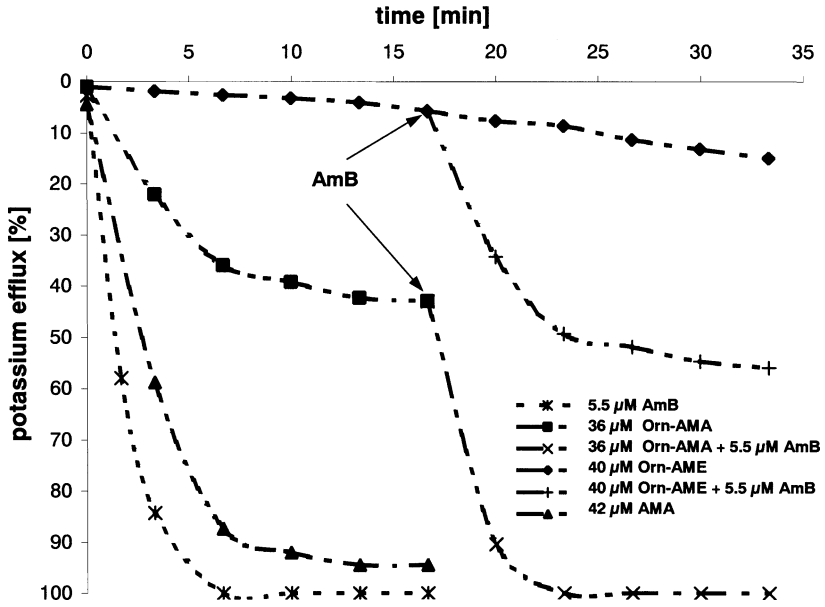
MIC—minimal compound concentration which completely inhibits fungal growth.
t_{50}—time of 50% of intracellular potassium release from fungal cells.
c—the compound concentration for which t_{50} value was obtained.

Table 3. The effect of examined compounds on human erythrocytes and degree of their self-association

			Erythrocytes			Self-association
Group	No	Compound	EK_{50} [μM]	EH_{50} [μM]	EH_{50}/EK_{50}	c_s [μM]
Reference	1	AmB	1.6	1.8	1.06	2.0
Amides	2	AMA*	13	23	1.75	36
	3	AMPA	10	n.hem.*	^	12
	4	APEA	24	73	3.12	9.35
	5	AMEA	20	25	1.25	12
	6	HAMA	31	n.hem.*	^	2
***N* – alkyl methyl esters**	7	SAME	34	153	4.5	12
	8	DMS-AME**	14	17	1.21	2
***N* – aminoacyl amides**	9	Orn-AMA	12	40	3.46	13
***N* – aminoacyl methyl esters**	10	Orn-AME	75	n.hem.*	^	>1.8

EK_{50}—the concentration of compound tested causing 50% of intracellular potassium release from human erythrocytes.
EH_{50}—the concentration of compound tested causing 50% hemolysis
n.hem.*—50% of hemolysis was not reached up to 100 μM concentration.
c_s—the concentration of compound tested in which $s = A_{348}/A_{409}$ equal to 1.

Fig. 2. Time course of potassium release from erythrocytes.



leakage as well as for its complete release was longer for the derivatives than for AmB.

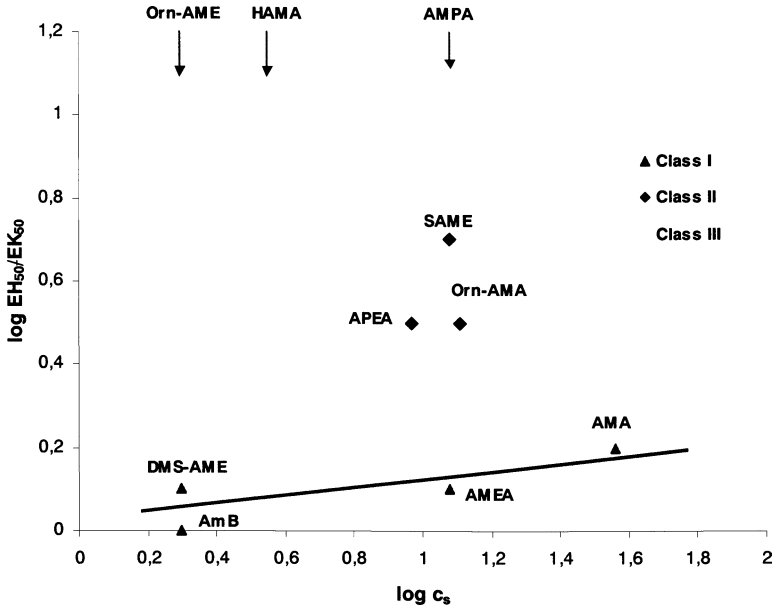
Red blood cells are routinely used as a mammalian cell model in toxicity determination of the polyene macrolide antibiotics. In these studies the toxicity of AmB derivatives toward human erythrocytes was monitored by their ability to induce intracellular potassium release and by hemolytic activity on human erythrocytes. The concentrations causing loss of 50% of intracellular potassium (EK_{50}) or hemoglobin (EH_{50}) were estimated from dose response curves. Values of EK_{50} and EH_{50} (Table 3) are included in wide range of concentration but indicate that all derivatives are definitely less toxic than the parent antibiotic. EK_{50} for Orn-AME was 40-fold, for SAME and HAMA 20-fold and for remaining derivatives about 10-fold higher than for AmB. Straight correlation between potassium efflux and hemolysis was not observed. Membrane permeabilization not always was followed by hemolysis. Only AMA, AMEA and DMS-AME induced potassium efflux and hemolysis at similar concentrations. For APEA, SAME and Orn-AMA hemolysis was observed at much higher concentrations than potassium release. AMPA, HAMA and Orn-AME practically were not hemolytic. It should be noted that permeabilizing efficiencies of these three compounds were different. It means, that lack of hemolysis not always reflected lack of membrane injury.

The kinetics of potassium efflux from erythrocytes induced by AMA, Orn-AMA and Orn-AME used at similar concentrations and AmB used at ten times lower concentration are shown in Fig. 2. Time course of potassium efflux induced by AMA was similar to that induced by AmB. In the case of Orn-AMA release of potassium was only partial and almost none in the case of Orn-AME. AmB added to cell suspension 16 min after Orn-AMA caused complete potassium loss (not shown). In contrast, AmB added after Orn-AME was not able to permeabilize whole cells population and induced only partial potassium loss has been observed. Similarly, partial cell permeabilization was observed when AmB was added some time after SAME (not shown).

Discussion

Although the mechanism of action of polyene macrolide antibiotic AmB on eukaryotic organisms has been well documented, the knowledge on the molecular basis of its selective toxicity is still insufficient. It is understandable, as it is difficult to uncover the mechanisms of selectivity of polyene macrolides using for the experiments, very poorly selective compound like AmB. It could be expected that the studies on AmB derivatives with much improved selective toxicity can lead to the recognition and exploitation for the rational drug design of so far “hidden” pharmacological

Fig. 3. The relationship between RBC permeability pathway characteristics and degree of self-association of AmB and its cationic derivatives.



potential of this otherwise valuable standard antifungal drug.

Many authors devoted their research to investigate the selective toxicity of monomeric and self-associated forms of AmB[6,7,9,30]. It has been shown that aggregation state of AmB affects its cholesterol/ergosterol affinity in model membranes[31] as well as fungal cells/mammalian cells selectivity[6,9,30]. AmB toxicity to red blood cells has been attributed to water soluble oligomers whereas monomeric form and insoluble aggregates have been considered as non-toxic[6,30]. It now seems to be established that monomeric form of AmB is of much higher selectivity than soluble aggregates. It was postulated that monomeric AmB is not able to create permeability pathways only in the cholesterol containing membranes. In contrast soluble aggregates of AmB are effective on cholesterol as well on ergosterol containing membranes[6].

In this paper we have investigated the group of positively charged, water soluble cationic derivatives of AmB (Table 1), with various selectivity and different tendency of self-association in aqueous medium, with the aim to evidence whether the monomer/self-associated species ratio is an essential factor for the selective toxicity of chemically modified AmB. The studied compounds are characterized also by the presence of different types of voluminous substituents at amino and carboxyl groups; this structural factor has been postulated to influence the selectivity of modified AmB[15,17~19].

The main conclusion of this studies is that unlike for AmB, the monomer/self-associated species ratio of the studied derivatives (Table 2) is not an essential in governing the selective toxicity of these compounds. Derivatives aggregating similarly to AmB (c_s=2) are definitely less toxic than parent antibiotic. AMA, derivative exhibiting the lowest tendency to self-association is not least toxic. It is also interesting to note that there is no correlation between two tests of toxicity against RBC used in these studies (Table 2). It might suggest that some derivatives induce permeability pathways of different characteristics, or that they exhibit different time course of cell membrane injury. According to EH_{50}/EK_{50} ratio the compounds studied could be divided into three classes (Fig. 3):

I. agents for which potassium efflux and hemolysis occur at similar concentration, $EH_{50}/EK_{50}\sim1$: AmB, DMS-AME, AMEA, AMA

II. agents for which potassium efflux occurs at significantly lower concentration than hemolysis, $EH_{50}/EK_{50}>3$: APEA, Orn-AMA, SAME

III. non-hemolytic agents inducing potassium efflux: AMPA, HAMA, Orn-AME

Our earlier results indicate that also another low toxic AmB derivative, MFAME belongs to class III[17,19].

Compounds belonging to above classes possess also different kinetics of potassium efflux (Fig. 2). AmB as well as AMA cause total potassium efflux within about 10 minutes. In the case of Orn-AMA (class II) the initial rate of potassium efflux is relatively high but the final release is only partial. The subsequent AmB addition results in complete potassium release. For class III compound (Orn-AME), the rate of potassium efflux is small and AmB addition results in partial release only. It should be stressed that type of permeability pathway do not depend on degree of self-association of the derivatives (Fig. 3).

The data obtained support our earlier assumption[15,17~19] that the structural factor, essential for the selective toxicity of AmB derivatives is the presence of a bulky substituent in the molecule, preferably located at the amino group of mycosamine moiety.

Acknowledgements

The authors acknowledge financial support of these studies by the Committee for Scientific Research (KBN) grant No 4 T09B 071 23 and in part by the Chemical Faculty, Gdansk University of Technology.

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