DIFFERENTIAL SELECTIVE TOXICITY OF DMS-AUREOFACIN COMPONENTS

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Trimethylammonium methyl esters (DMS) of polyene macrolides are products of the methylation of native polyene antibiotics with dimethyl sulfate. We isolated individual components of the DMS-aureofacin complex and characterized their toxicity and activity to induce permeability changes in cell membranes. The DMS-aureofacin complex contained five components readily separated by thin-layer chromatography on polygram cellulose plates. DMS-Aureofacin A, a major component of the complex (90%), showed poor selective toxicity between yeast cells and mammalian cells grown in culture. DMS-Aureofacin B (6%) and DMS-aureofacin E (4%) exhibited very high biological activities and differed qualitatively in selective toxicity. DMS-Aureofacin B was much more active for mammalian cells than for yeast cells. In contrast, DMS-aureofacin E was much more active for yeast cells than for mammalian cells. DMS-Aureofacin C and D were present in the complex in only minute quantities which did not permit their biological characterization.

Chemical modifications of polyene macrolide antibiotics have been developed to increase quantitative differences in their affinity to molecular targets in pathogenic and host eucaryotic cells, namely, different sterols located in plasma membrane[1]. Another major reason for the derivatization of polyene antibiotics is to alter the water insolubility of the native compounds which limits the application of these drugs.

Several groups of chemically modified polyene macrolide antibiotics with improved selective toxicity and water solubility have been reported: 1) water soluble *N*-acyl derivatives exhibit improved selective toxicity, although their biological activity is diminished[2,3]; 2) methyl esters show markedly improved selective toxicity, but their soluble hydrochlorides are not stable[4,5]; 3) water soluble *N*-methylglucamine salts of *N*-glycosyl derivatives are effective in the treatment of systemic mycoses, but they are less active than native polyenes[6]; 4) water soluble salts of amides provide large variations of selective toxicity depending on the polyene used and the type of amide residue introduced[7]; 5) methyl esters of trimethylammonium derivatives (DMS) exhibit markedly improved selective toxicity, high biological activity and good water solubility[8~10]. Recently reported D-ornithyl amphotericin B methyl ester shows good water solubility, high activity against *Candida* but low selective toxicity when used *in vitro*[11].

DMS-Aureofacin, a representative member of the trimethylammonium methyl ester derivatives of polyene macrolides[8], is of special interest because of its unique features such as very high biological activity[8,9]; good selective toxicity[8]; a broad antimicrobial spectrum [8~10,12]; water solubility; potentiation capacity expressed by a high dissociation index between permeabilizing and toxic effects induced[9]; high specificity of membrane changes, allowing for full recovery of mammalian cells after treatment with polyene concentrations inducing an efflux of 90% of intracellular potassium[10]. These features make DMS-aureofacin a prime candidate for chemotherapeutic use. DMS-Aureofacin, as well as other

DMS derivatives of polyenes, is the product of methylation of native polyene macrolide antibiotics with dimethyl sulfate[8]. It has been postulated that the products of the reaction have methyl esterified carboxyl groups and a quaternized nitrogen atom of amino sugar. Also, an aromatic amino group (if present) can be substituted with one or two methyl groups[8]. Biological effects of DMS derivatives of polyene macrolides have been studied with the product obtained after the reaction of polyene macrolide with dimethyl sulfate, without isolation of selective compounds which varied with degree of methylation[8~10,12]. In this study we report the biological properties of isolated individual components of DMS-aureofacin.

Materials and Methods

Synthesis of DMS-Aureofacin

DMS-Aureofacin was synthesized from the aromatic heptaene aureofacin[13] (Polish United Pharmac, Ind., POLFA, Poland) according to the method of FALKOWSKI *et al.*[8] with the following modifications: methylation was carried out at 34°C for 3 hours, DMS-aureofacin was converted into the chloride form by washing the *n*-butanol extract of the reaction products with an aqueous solution of 3% KCl. The purity of DMS-aureofacin obtained was about 90%, as calculated from $E^{1\%}_{1cm}$ value measured at 379 nm.

Isolation of DMS-Aureofacin Components

The components of DMS-aureofacin were isolated by preparative thin-layer chromatography (TLC) on polygram cellulose 300 PEI/UV plates (Brinkman Instruments, Westbury, NY). DMS-Aureofacin was dissolved in MeOH at a concentration of 10 mcg/ml. The sample was spotted on PEI plates (20 ×20 cm) and the components were separated in a solvent system consisting of $CHCl_3$ - MeOH - *n*-butanol - *n*-hexane - H_2O - acetone (1: 1: 1: 1: 1: 3). After drying the plates, spots were located by long wave UV light. Different components of DMS-aureofacin were collected separately by scraping the individual spots from the plates. About 1 mg of cellulose containing the individual components was collected from 20 plates and polyene antibiotic was eluted 3 times with 2 ml of MeOH. All eluates were collected, dried under nitrogen at 25°C, and diluted to 0.5 ml. The concentration of each DMS-aureofacin component in the methanol solution was calculated from $E^{1\%}_{1cm}$ values measured at 379 nm. Methanol solutions of DMS-aureofacin and its components were stored at −20°C in the darkness and were used for biological activity assays within 48 hours after isolation.

Cells and Media

Baby hamster kidney (BHK-21) cells were grown in shaken culture in serum-free modified Waymouth 752/1 medium[14]. Mouse L cells were grown in shaken culture in modified Waymouth 752/1 medium[15], supplemented with 5 mcg/ml sodium oleate. Monkey kidney (LLC-MK2) cells were grown in a monolayer culture in Eagle's minimum essential medium supplemented with 5% newborn calf serum. All cells were incubated at 37°C.

Saccharomyces cerevisiae strains 28382 and 28383 were grown in Bacto YM broth (Difco Laboratory, Detroit, MI) and the same medium supplemented with ergosterol (10 mcg/ml). Cells were incubated in a shaken culture at 28°C.

Measurement of Polyene Macrolide Activity Inducing Potassium Leakage

Biological activities of polyene macrolides were determined by their ability to release 50% potassium from cells (K_{50}) as described earlier[9]. Cells taken from the logarithmic phase of growth were centrifuged and resuspended in HANKS' balanced salt solution (BSS) at a concentration of 0.5 mg cell protein per ml. The cell suspension was distributed into 2 ml samples and the desired amount of polyene was quickly mixed with the samples. The final amount of methanol in the reaction mixture did not exceed 1% which had no effect on membrane permeability of all tested cells. Cells were incubated for 30 minutes at 30°C, centrifuged for 5 minutes at 250×*g* and washed with K^+-free medium as described earlier[9]. After washing, *S. cerevisiae* cells were boiled with 0.5 ml of 1 N HCl for 10 minutes, and

4.5 ml of water was added; BHK-21, mouse L and LLC-MK2 cells were suspended directly in water[9]. The amount of potassium released from cells was measured by atomic absorption as described earlier[9].

Toxic Effect of Polyene Macrolide Antibiotics on Cells

BHK-21 and *S. cerevisiae* (strain 28382) cells were used for the toxicity test. Cells taken from the logarithmic phase of growth were suspended in BSS at the density required to obtain 0.5 mg of cell protein per ml. Cells were incubated with various concentrations of polyene macrolide antibiotics for 30 minutes at 30°C. Cells not treated with the antibiotic and incubated under the same conditions were used as a control. After the incubation, the cells were sedimented for 5 minutes at 250×*g*, washed once with BSS and resuspended in polyene antibiotic-free medium. This cell suspension was used to determine cell survival. The amount of BHK-21 cells surviving after treatment with polyene macrolide was determined by the cloning technique described earlier[10]. The survival of *S. cerevisiae* cells after treatment with polyene antibiotics was determined as follows: Ten-fold dilutions were made from the cell suspension and 0.1 ml of each dilution was spread evently on the surface of agar—growth medium prepared in petri dishes (5 cm in diameter). After 4 days incubation at 28°C, the number of *S. cerevisiae* colonies was counted and compared with the number of colonies obtained from control cell suspensions. The data obtained were used to determine the concentration of polyene antibiotic reducing the number of colonies by 50% (TC_{50}).

Results and Discussion

The methylation process of aureofacin, performed under the conditions described above, was completed within 3 hours and no free aureofacin could be detected by TLC of the reaction mixture. The DMS-aureofacin reaction mixture contained at least five different components which could be distinguished by differences in Rf values (Table 1). Three major components were readily isolated and characterized: DMS-aureofacin A, DMS-aureofacin B and DMS-aureofacin E (Table 1). Based on data

Table 1. Characterization of DMS-aureofacin components.

Antibiotic	Rf*1	Amount*2
DMS-Aureofacin A	0.95	90
DMS-Aureofacin B	0.90	6
DMS-Aureofacin C	0.80	<1
DMS-Aureofacin D	0.75	<1
DMS-Aureofacin E	0.70	4
Aureofacin*3	0.55	

*1 Rf values obtained from the separation of DMS-aureofacins on polygram cellulose PEI/UV in the solvent system consisting of $CHCl_3$ - MeOH - *n*-butanol - *n*-hexane - H_2O - acetone (1 : 1 : 1 : 1 : 1 : 3).

*2 Percentage participation of each DMS-aureofacin component calculated after separation of 2 mg of DMS-aureofacin mixture on 20 PEI/UV plates and elution from cellulose by MeOH as described in Materials and Methods. The amount of DMS-aureofacin and its components were calculated from $E^{1\%}_{1cm}$ values measured at 379 nm.

*3 Aureofacin is a complex of two heptaenes: vacidin A and gedamicin, which differ only with the presence or absence of a methyl group in the aromatic fragment of the polyene molecule[17]. These two components form one spot on PEI/UV cellulose when developed in the above solvent system.

Fig. 1. Ultraviolet-visible spectra of DMS-aureofacin A (A), DMS-aureofacin B (B) and DMS-aureofacin E (E) as chloride salts in MeOH.

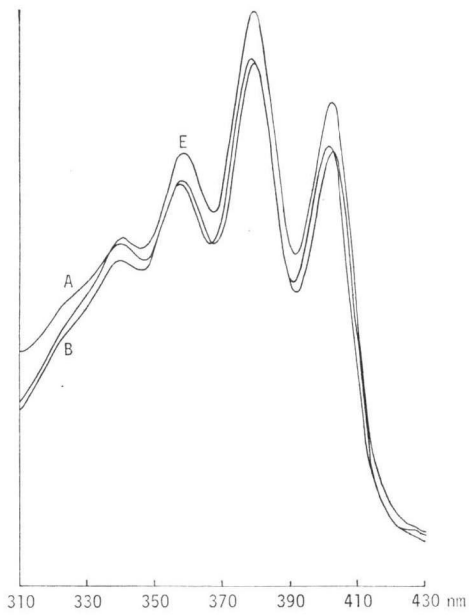

Table 2. The effect of DMS-aureofacin and its components on plasma membrane permeability changes in mammalian and yeast cells.*1

Antibiotic	Concentration of polyene antibiotic (mcg/ml) releasing 50% of intracellular K^+ (K_{50})				
	BHK-21	LLC-MK2	Mouse L	*S. cerevisiae*	
				Strain 28382	Strain 28383
DMS-Aureofacin*2	0.2	0.4	0.2	0.3	0.3
DMS-Aureofacin A	3.5	3.0	2.8	2.8	3.0
DMS-Aureofacin B	0.05	0.08	0.05	2.5	3.0
DMS-Aureofacin E	3.0	2.5	3.5	0.05	0.06
Aureofacin*3	0.05	0.1	0.08	0.05	0.05

*1 Cells taken from the logarithmic phase of growth, as described in Materials and Methods, were suspended in BSS (pH 7.2) at an appropriate density to obtain 0.5 mg of cell protein per ml suspension. The cell suspension was divided into 2 ml samples and 20 μl of the polyene antibiotic solution was added to each sample to obtain the desired concentration of polyene macrolide. Cells were incubated with the antibiotic for 30 minutes at 30°C in a shaker water bath. Potassium released from the cells was measured as described in Materials and Methods.

*2 DMS-Aureofacin mixture used for the separation of the individual components.

*3 Parent polyene antibiotic used for derivatization.

Table 3. Toxic effect of DMS-aureofacin and its components on BHK-21 and *S. cerevisiae* cells.*1

Antibiotic	Concentration of polyene antibiotic (mcg/ml) reducing the number of cells surviving by 50% (TC_{50})	
	BHK-21	*S. cerevisiae* (strain 28382)
DMS-Aureofacin*2	5.0	3.5
DMS-Aureofacin A	5.0	5.0
DMS-Aureofacin B	2.0	10.0
DMS-Aureofacin E	10.0	1.0
Aureofacin*3	0.5	0.5

*1 Cells taken from the logarithmic phase of growth were suspended in BSS (pH 7.2) at an appropriate density to obtain 0.5 mg of cell protein per ml of suspension. Cells were treated with different concentrations of polyene for 30 minutes at 30°C in a shaker water bath. The cells were then sedimented by centrifugation for 5 minutes at 250×*g* at 25~30°C, washed with BSS and resuspended in polyene-free medium. The number of cells surviving the polyene treatment was determined as described in Materials and Methods.

*2 DMS-Aureofacin mixture used for isolation of the components.

*3 Substrate taken for the DMS-derivatization.

obtained (Table 1), we could estimate that the relative percentages of DMS-aureofacin A, DMS-aureofacin B and DMS-aureofacin E were 90%, 6% and 4%, respectively. DMS-Aureofacin C and DMS-aureofacin D were present in the reaction mixture at quantities below 1%. All isolated components of DMS-aureofacin exhibited identical UV spectra, typical for aromatic heptaenes with major absorption maxima at 359, 379 and 402 nm, as shown in Fig. 1. The biological properties of the components of DMS-aureofacin were determined for mammalian cells and *S. cerevisiae* (Tables 2 and 3). The results obtained indicated that the components of DMS-aureofacin differ substantially in biological activity as well as in selective toxicity. DMS-Aureofacin A, the major component of the mixture, had very low biological activity and little or no selective toxicity when used for the treatment of animal and fungal cells (Tables 2 and 3).

Very interesting biological properties were exhibited by two minor components isolated from DMS-aureofacin. DMS-Aureofacin B demonstrated from 50 to 60 times higher affinity for animal cells containing cholesterol in their

plasma membranes than for *S. cerevisiae* containing ergosterol (Table 2). Also, great dissociation was observed between the concentrations of DMS-aureofacin B inducing permeabilizing and toxic effects on BHK-21 cells (Tables 2 and 3). This high dissociation index (TC_{50}: K_{50}=40) demonstrated the specificity of the membrane permeability changes induced by this compound in BHK-21 cells[9]. The conditions used in the cloning procedure were favorable for the repair of polyene-induced permeability changes in the cell membrane[10]. BHK-21 cells, which lost almost all intracellular K^+ after 30 minutes of the treatment with DMS-aureofacin B (data not shown), were able to regain their intracellular K^+ level and to repair polyene-induced damage in the membranes when cells were transferred into antibiotic-free medium. The reversible permeability changes in cell membranes induced by polyene macrolides can facilitate the membrane transport of other agents, thus potentiating their effects on cells[1]. The dissociation between the concentration of a polyene macrolide inducing membrane permeability changes and the toxic concentration has been suggested as an indication of the potentiation capability of the antibiotic[16]. Therefore, DMS-aureofacin B with a dissociation index of 40 seems to be a good candidate as a potentiating agent with very low toxicity to mammalian cells (Tables 2 and 3).

DMS-Aureofacin E exhibited a high selective affinity index towards *S. cerevisiae* when compared with mammalian cells. From 50 to 70 times lower concentrations of this compound were required to release 50% of the cell K^+ from *S. cerevisiae* than from mammalian cells (Table 2). Contrary to DMS-aureofacin B, DMS-aureofacin E demonstrated a high dissociation index for *S. cerevisiae* (TC_{50}: K_{50}= 20) and a low index for BHK-21 cells (Tables 2 and 3). These observations implied higher affinity of DMS-aureofacin E towards yeast cells containing ergosterol in their membranes than toward mammalian cells containing cholesterol, which suggests that this compound may be an antifungal agent with low animal toxicity.

Elucidation of the chemical structure of DMS-aureofacin isolated components is in progress. In our preliminary experiments, DMS-aureofacin A showed the strongest basic character of all components of DMS-aureofacin. DMS-Aureofacin B and DMS-aureofacin E were separately removed from the carboxymethyl cellulose column with a solvent system consisting of MeOH - H_2O (1: 2); however, DMS-aureofacin A could be removed from the column only when 1 M NaCl was added to the solvent mixture.

Because both DMS-aureofacin C and DMS-aureofacin D were present in the reaction mixture in very low quantities, we were not able to isolate substantial amounts of those compounds to test their activities. The isolated mixture of DMS-aureofacin B and DMS-aureofacin C showed decreased activity in releasing K^+ from BHK-21 and yeast cells when compared to the activity of DMS-aureofacin B alone. Similarly decreased activity of DMS-aureofacin E was monitored when this compound was isolated along with DMS-aureofacin D.

These results show that DMS-aureofacin contains three major components which account for biological properties of this polyene macrolide antibiotic. Since aureofacin is a mixture of vacidin and its methyl derivative (gedamicin), it is important to emphasize that for the first time, polyene antibiotics qualitatively differing in their selective activity were obtained from the same parent compound (vacidin) as products of a single chemical reaction. Therefore, DMS-aureofacin B and DMS-aureofacin E are ideal candidates to study the molecular aspects of the selective toxicity of polyene macrolide antibiotics on fungal and mammalian cells.

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