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The Mechanism of Overcoming Multidrug Resistance (MDR) of Fungi by Amphotericin B and Its Derivatives

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Abstract Comparative studies were performed to determine the activity and cytotoxicity of amphotericin B (AmB) and its derivatives on standard strain of *Saccharomyces cerevisiae* and its transformants with cloned genes from *Candida albicans* encoding multidrug resistance (MDR) pumps of ATP-binding cassette and major facilitator superfamilies. The AmB derivatives: amphotericin B 3-dimethylaminopropyl amide and *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester were shown to be fungistatic and fungicidal towards MDR strains, by membrane permeabilization mechanism. Antibiotic-cell interaction monitored by energy transfer method indicates similar membrane affinity in parent strain and its MDR transformants. Experiments with fungal cells loaded with rhodamine 6G point to lack of competition between this dye and AmB and its derivatives for efflux driven by CDR2p. It can be thus assumed that AmB and its derivatives overcome fungal MDR by not being substrates of the multidrug exporting pumps, presumably due to their large molecular volumes.

Keywords amphotericin B, amphotericin B derivatives, antifungal activity, multidrug resistance

Introduction

Antifungal treatments against systemic infections are hampered by several factors including the limited number of available active and nontoxic drugs and the development of resistance, especially multidrug resistance (MDR) [1~6].

Among the mechanisms underlying the MDR phenomenon, the most important is the action of transmembrane proteins that catalyze the active efflux of cytotoxic xenobiotic compounds from the cells. A diminution of the concentration of active compounds at the target site contributes to the drug ineffectiveness [2, 3, 5, 6].

Multidrug transporters can accept a broad range of compounds that are variable in structure as well as mode of action [7, 8] and cover most of the antifungal agents used in clinics for the treatment of disseminated infections [1, 9]. The structural factors determining the substrate properties of antimycotics for multidrug transporters have not been as yet identified, however the strong size dependence for xenobiotic efflux has been observed for yeast multidrug transporter Pdr5 [10].

The MDR transporting proteins in fungi are usually classified according to the energy source supporting action of the efflux pump. One group belongs to the ATP-binding cassette superfamily (ABC) for which the energy is supplied by ATP hydrolysis. Another group, the major facilitator superfamily (MFS), utilizes proton motive force. The MFS transporters work by antiport mechanism and efflux out of the cell is driven by exchange for H^+ ions

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[11].

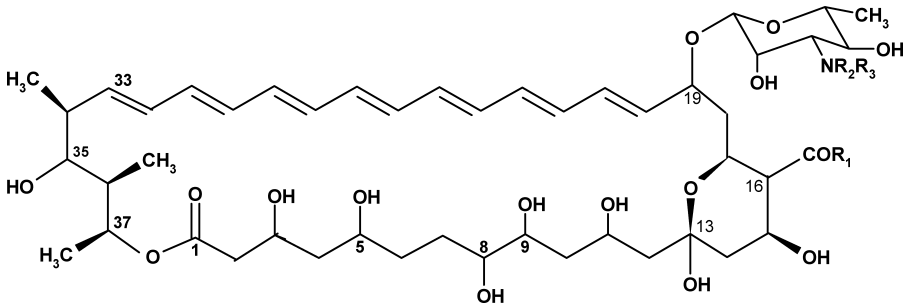
A number of MDR transporters representing both families have been already identified in fungi. Well characterized are ABC transporters, Cdr1p and Cdr2p in *Candida albicans* [12~14], Pdr5p in *Saccharomyces cerevisiae* [15] and CaMDR1p, a MFS transporter of *C. albicans* [16~18].

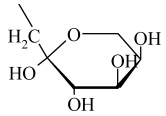
Amphotericin B (AmB), however rather toxic [19], for decades has been used clinically as a life-saving drug for the treatment of systemic mycoses, due to its other positive features [20], and the ability to overcome fungal MDR [21, 22]. Little is known about the ability to overcome this type of resistance by AmB derivatives designed to reduce the drug toxicity. Studies of AmB and its derivative *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (MFAME) on *S. cerevisiae* JG 436 and its *CDR1*-containing mutant (PS 12-4) evidenced that both compounds induced comparable stimulatory, permeabilizing and fungicidal effects in both fungal organisms [22]. However, the mechanism of overcoming MDR by AmB and its derivatives remains unknown. It is of importance to evidence whether chemical modification of AmB affects the ability of the compound to overcome MDR, what is often the case with many other active agents. AmB and derivatives are membrane active compounds inducing in sterols containing cells lethal permeability changes to different extent in various organisms, differing in regard to lipid components of plasma membranes [19, 23]. Chemical modifications of AmB are aimed at the increase of differential effects on fungal *vs.* mammalian membranes. Consequently, this could affect the membrane localized drug effluxing pumps in MDR strains.

It should be noted that native, as well as chemically modified AmB, besides binding to cell membrane also undergo internalization [24]. Therefore examining the ability of these compounds to overcome MDR various effects should be taken into account: i) inhibition of MDR genes expression at various stages; ii) inhibition of posttranslational transporter proteins modification; iii) interference with transporter proteins ATP binding domains; iv) irreversible inactivation of transporters proteins; v) lack of substrate properties (affinity) for drug exporting pumps.

In the present studies we examined the antifungal action of AmB and its two semisynthetic derivatives amphotericin B 3-dimethylaminopropyl amide (AMA) and MFAME (Table 1) against standard strain of *S. cerevisiae* and its mutants with cloned genes from *C. albicans* encoding

Table 1 Structures and biological properties of amphotericin B (AmB) and derivatives



Compound	R_1	R_2	R_3	MIC [μM]	EK_{50} [μM]	EH_{50} [μM]
AmB	–OH	–H	–H	0.05	1.2	1.95
AMA	$-NH(CH_2)_3N(CH_3)_2$	–H	–H	0.08	13	23
MFAME	$-OCH_3$	$-CH_3$		0.3	>100	>250

AMA: amphotericin B 3-dimethylaminopropyl amide.

MFAME: *N*-methyl-*N*-D-frutopyranosylamphotericin B methyl ester.

MIC: Minimal compound concentration that completely inhibits fungal growth in liquid medium (*Candida albicans* ATCC 10261) determined by serial dilution method.

EK_{50}: The compound concentration causing 50% of intracellular potassium release from human erythrocytes.

EH_{50}: The compound concentration causing 50% release of hemoglobin from human erythrocytes.

MDR pumps of ABC and MFS families. The compounds selected for these studies are of varied selective toxicity: poor (AmB), medium (AMA) and high (MFAME). In this research we wanted to elucidate whether the modification of AmB molecule aimed at the development of nontoxic compound affects its activity against resistant fungi and if so what is the mechanism of overcoming fungal MDR by AmB and its derivatives. The experiments performed comprised determinations of: i) antifungal activity (MIC and MFC); ii) permeabilizing efficiency (dissipation of H^+ and K^+ gradients); iii) membrane binding efficiency and binding stability (by energy transfer method using fluorescent membrane probe); iv) effect of AmB and derivatives on efficacy of transporters mediated rhodamine 6G (R6G) efflux. Experiments aimed at investigation of substrate properties of examined antibiotics for MDR transporters were aided by calculation of their molecular volumes, the factor known to affect the transporter proteins mediated xenobiotics efflux.

Materials and Methods

Chemicals

1-(4-trimethylammoniumphenyl)6-diphenyl-1,3,5-hexatriene (*p*-toluenesulfonate salt) (TMA-DPH), DMSO, R6G, uracil, glucose, ammonium sulfate and yeast synthetic drop-out medium supplement were purchased from Sigma-Aldrich. Bacto - peptone, yeast extract and agar were Difco products.

Polyene Antibiotics

AmB was Sigma-Aldrich product. AMA and MFAME (L-aspartic acid salts) were synthesized in the Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology, Poland, according to the previously described procedures [25, 26]. Stock solutions of the compounds were prepared in DMSO just before use. Purity of the substances was determined by electronic absorption of their methanolic solutions (ε_{408}= 160000 $M^{-1} cm^{-1}$). All concentrations of the antibiotics given in the text correspond to 100% purity.

Human Red Blood Cells—Determination of Potassium Release and Hemolysis

Erythrocytes isolation from human blood as well as determination of permeabilizing activity and extent of hemolysis were carried out according to the previously described procedures [27].

Fungal Strains, Media and Growth Conditions

Fungal strains used were: *C. albicans* ATCC 10261; *S. cerevisiae* AD1234568 and its transformants, designated as ADCDR2 and ADCaMDR1, with cloned MDR *C. albicans* genes: *CDR2* and *CaMDR1*, respectively. *S. cerevisiae* AD1234568 was kindly provided by A. Goffeau, Université Catholique de Louvain, Belgium. In this strain seven ABC transporters genes, namely: *PDR5*, *PDR10*, *PDR11*, *PDR15*, *SNQ2*, *YCF1* and *YOR1* are deleted [28]. The multidrug resistant strains, ADCDR2 [29] and ADCaMDR1 [30], were from the collection of R. Prasad, School of Life Sciences, Javaharlal Nehru University, New Delhi, India.

S. cerevisiae standard strain (AD1234568) was maintained on slants containing: YNB v/o amino acids 1.7%, glucose 2.0%, ammonium sulphate 0.5%, uracil 0.003%, Yeast Synthetic Drop-out Medium Supplements (mixtures of amino acids) 1.92 g/liter and agar 2.0% while the transformants were maintained in similar medium but without uracil. *C. albicans* ATCC 10261 was maintained on agar slants composed of bacto - peptone 1.0%, yeast extract 1.0%, glucose 2.0% and agar 2.0%. The cells were multiplied in the media in which agar was omitted. The growth temperature for all strains was 30°C.

Determination of MIC and MFC

MICs were determined by the two-fold serial dilution method in proper liquid medium (YNBG–*S. cerevisiae*; BG–*C. albicans*) supplemented with NaCl 0.5% according to the procedure described earlier [27]. The lowest concentration of the antibiotic yielding no growth after 48 hours of incubation at 30°C was defined as MIC. For MFC determination 20 μl aliquots taken from samples in which cell growth was inhibited were transferred into fresh, growth liquid medium containing NaCl 0.5%. The lowest concentration of the antibiotic preventing visible growth after 48 hours incubation at 30°C was defined as MFC.

Determination of Potassium Efflux

Cells from 18 hours cultures were harvested by centrifugation (10 minutes, 3000×*g*), washed three times with saline and resuspended in saline at concentration 2.0 mg dry wt/ml. When the recorded signal had stabilized (10～15 minutes), the antibiotic solution was added and potassium level in suspension was continuously monitored during 30 minutes by potassium selective electrode connected to pH-ionmeter CPI-501, Elmetron. The amount of potassium released was expressed as a percentage of that recorded for the boiled control cell suspension (100% of potassium released).

Determination of H^+/K^+ Exchange

Cells from 18 hours culture were harvested by centrifugation (10 minutes, 3000×*g*), washed three times with deionized water and resuspended in water at concentration 2.0 mg dry wt/ml (1.0 ml of cell suspension, OD_{660} ~1, corresponded to 0.5 mg dry wt.). The initial pH ~6 of cell suspension was adjusted with 0.01 M HCl. The pH of cell suspension was measured with pH-meter CPI-551, Elmetron. After pH reading stabilization (~15 minutes), the experiment was started by addition of the antibiotic at final concentration 10 μM. Changes in proton and potassium concentrations were monitored simultaneously with pH and potassium selective electrodes respectively.

Cells Labeling and Fluorescence Measurements

The assay for antibiotic-cell interaction was carried out as described earlier by Szlinder-Richert *et al.* [24]. The fluorescence measurements were made on Perkin-Elmer LS-55 spectrofluorimeter using optically clear quartz cuvettes, 1 cm in diameter. All experiments were perfomed in 0.1 M KCl as a medium. Energy transfer efficiency (E) between fluorescent probe TMA-DPH incorporated into plasma membrane (donor) and polyene antibiotic (acceptor) was calculated using the formula described previously by Haynes *et al.* [31].

Determination of cfu

Fungal cells viability in the conditions of fluorescent measurements was checked by colony forming units determination. Cell suspension after preincubation (30 minutes, 37°C) with TMA-DPH and antibiotic at concentration used in energy transfer measurements, were diluted and plated on proper solid medium supplemented with 0.1 M KCl. After 96 hours of incubation at 37°C colonies were counted. cfu in treated cell suspensions were compared to control ones.

Antibiotics Binding Stability

The experiments were performed in two following variants: 1) cells suspended in 0.1 M KCl were preincubated with 2.0 μM TMA-DPH for 20 minutes in 37°C, then cells were separated by centrifugation (10 minutes, 3000×*g*), washed and resuspended in fresh 0.1 M KCl (10^6 cells/ml); when the fluorescence intensity had reached steady-state level (after ~5 minutes), 2.0 μM solution of antibiotic and after 15 minutes 50 mM solution of glucose were added; fluorescence intensity as a function of time was recorded; 2) cells suspended in 0.1 M KCl were preincubated with antibiotic (2.0 μM) and TMA-DPH (2.0 μM) for 20 minutes in 37°C, then washed, resuspended in fresh 0.1 M KCl and changes in fluorescence intensity over time were recorded in the presence or absence of glucose as an energy source.

Measurement of R6G Uptake and Glucose-induced Efflux

The rhodamine efflux was determined using a protocol described by Maesaki *et al.* [32] with few modifications. Briefly, yeast cells were pregrown overnight in YNBG broth. Cells (10^8 cells/ml) were transferred to fresh medium and allow to grow for 4 hours at 30°C; they were then resuspended in glucose free 0.1 M KCl at a cell density of 10^8 cells/ml and incubated at 30°C with shaking (150 rpm) for 1 hour. R6G was subsequently added at a final concentration of 10 μM and and incubated in the darkness at 30°C for 10 minutes (till the dye showed a steady intracellular level of accumulation). After incubation R6G-loaded cells were quickly spun down (3 minutes, 9000×*g*), washed once with 0.1 M KCl and finally resuspended in 0.1 M KCl. In the experiments aimed at the determination of the effects of AmB and derivatives on the efflux of R6G from fungal cells polyene antibiotic solution in DMSO was added (final concentration - 2.0 μM) to the cell suspension. These experiments were performed in the absence and in the presence of glucose (2.0%). The duplicate 2.0 ml samples were withdrawn from both suspensions (with and without glucose) at time zero (immediately after addition of glucose), samples were centrifuged at 9000×*g* for 3 minutes. The resulting supernatant was collected and absorption was measured at 527 nm on Perkin Elmer, λ 45, UV-VIS spectrophotometer. After 10, 20, 40 and 50 minutes of further incubation the 2.0 ml samples were withdrawn again, cells were removed by centrifugation and absorbance of R6G extruded in the supernatant was measured. The concentration of R6G was calculated using a standard concentration curve of R6G. In all experiments glucose-free controls as well as controls without antibiotics (containing appropriate amounts of solvent instead antibiotics) were included.

Calculation of Molecular Size Parameter

Molecular volumes were calculated as a volume of the solvent excluded surface. The standard set of van der Waals radii was employed. Initial models of antibiotic molecules were built basing on the crystal structure of AmB [33]. The obtained structures were then subjected to conformational analysis using force field method implemented in QUANTA package (Molecular Simulations Inc., San Diego, 2000). The lowest energy geometries, additionally optimized at the semiempirical level of quantum chemistry, were taken to volume calculation.

Results and Discussion

Selective Toxicity Characteristics

The AmB derivatives studied 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 (Table 1). This type of structural modification of AmB changed lipophilic/hydrophilic balance and net charge of the molecule. AmB is zwitterionic whereas MFAME and AMA are positively charged at physiological pH. Both derivatives form water soluble salts.

The selective toxicity of AmB and derivatives was determined side by side on *C. albicans* and human erythrocytes, chosen as the representatives of fungal pathogenic and host cells, respectively (Table 1). It was found that in comparison with parent AmB, derivatives exhibit few fold lower antifungal activity and diversified toxicity towards human erythrocytes. In case of AMA improvement of selective toxicity was a few-fold whereas MFAME exhibited dramatic reduction of toxicity (~100 fold) and was not hemolytic.

Action on *Saccharomyces cerevisiae* and Its MDR Transformants

Antifungal Activity

As a measure of antifungal activity of AmB and derivatives on standard strain of *S. cerevisiae* and its MDR transformants: ADCDR2 and ADCaMDR1, MIC (cytostatic effect) and MFC (cytotoxic effect) were determined. The results obtained (Table 2) indicate that transformant containing gene encoding MFS transporter has the same sensitivity as a parent strain to compounds tested. The strain containing ABC extrusion pump is slightly less sensitive. The cytotoxic concentrations of AmB, AMA and MFAME were two-fold higher than cytostatic ones for parent strain as well as for both transformants. These results evidence that effectivity of AmB and derivatives remains practically unchanged for strains with encoded MDR genes of ABC and MFS families.

Table 2 Sensitivity of *Saccharomyces cerevisiae* standard strain and its MDR mutants to AmB, amphotericin B 3-dimethylaminopropyl amide (AMA), *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (MFAME)

Strain	Compound	MIC [μM]	MFC [μM]
AD1234568	AmB	0.1	0.27
	AMA	0.2	0.4
	MFAME	0.4	0.8
ADCDR2	AmB	0.27	0.54
	AMA	0.4	0.4
	MFAME	0.8	0.8
ADCaMDR1	AmB	0.1	0.27
	AMA	0.2	0.4
	MFAME	0.4	0.8

Cells were incubated with antibiotic for 48 hours at 30°C. Optical density of the cell suspension was determined at λ=660 nm.

Permeabilizing Effects

The membrane permeability alteration induced by AmB and both derivatives in the strains studied were compared by determination of: potassium efflux and potassium/proton exchange in the cells suspended in saline and distilled water, respectively.

Potassium Efflux

AmB and both derivatives (data not shown) at 10 μM concentration cause potassium release up to 70% after 30 minutes incubation. In order to compare the permeabilizing efficiency of the antibiotics studied, times required for 30% of intracellular potassium loss (t_{30}) were estimated (Table 3). Similarity of the potassium efflux and lack of significant differences in the t_{30} values indicate comparable efficiency of the antibiotics and similar sensitivity of the strains studied.

H^+/K^+ Exchange

Time courses of H^+/K^+ exchange induced by AmB, AMA and MFAME for the strains studied are shown in Fig. 1. The results obtained suggest coupling between intracellular potassium efflux and external proton uptake. Permeabilizing action of AmB and derivatives on standard strain and on transformants were practically the same.

Affinity to Cells

The affinity of AmB and the derivatives to standard strain of *S. cerevisiae* and its MDR transformants was studied by fluorescence method based on the energy transfer between

Table 3 The comparison of t_{30} values obtained for *S. cerevisiae* standard strain and its MDR mutants

Compound	t_{30} [minutes]		
	AD1234568	ADCaMDR1	ADCDR2
AmB	12.5±1.7	15.0±1.4	10.8±1.8
AMA	12.0±0.9	10.2±1.3	9.3±1.3
MFAME	11.8±0.4	10.5±1.4	10.0±0.8

t_{30}: Time of 30% of intracellular potassium release from fungal cells treated with antibiotic at 10 μM concentration.

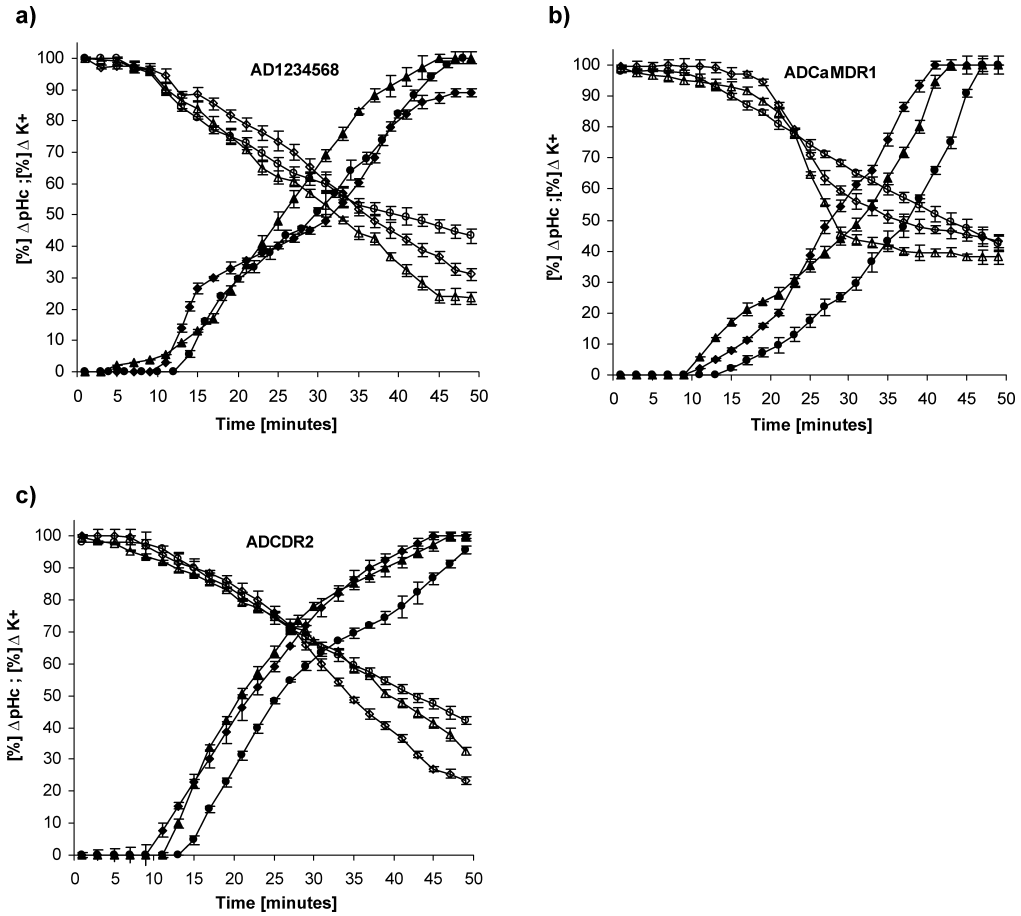


Fig. 1 Kinetics of H^+/K^+ exchange in *Saccharomyces cerevisiae*: a) AD1234568, b) ADCaMDR1, c) ADCDR2 induced by 10 μM amphotericin B (AmB), amphotericin B 3-dimethylaminopropyl amide (AMA) and *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (MFAME).

Yeast cells (2.0 mg dry wt/ml) were suspended in distilled water. Symbols: ◆ H^+/AmB; ▲ H^+/AMA; ● H^+/MFAME; ◇ K^+/AmB; △ K^+/AMA; ○ K^+/MFAME.

membrane fluorescence probe TMA-DPH (donor) and the polyene antibiotic (acceptor). Previously, this method was used to investigate the binding of AmB and some derivatives to mammalian (renal tubular cells, thymocytes, leukemia), fungal and bacterial cells [24, 31, 34, 35]. Fluorescent measurements were performed with resting and metabolizing cells. Resting cells experiments were performed in cell suspension in 0.1 M KCl. This medium was used because in the presence of antibiotics the number of the viable cells was drastically reduced in saline whereas in 0.1 M KCl cells remained viable during the course of experiment (Table 4). Essential differences in viability observed in both media could be explained in terms of protective action of potassium ions. In potassium chloride the concentration of K^+ ions is comparable to the concentration in fungal cytoplasm and as a consequence potassium leakage is prevented enabling to maintain the metabolic equilibrium. It should be stressed that permeabilizing action of antibiotic is the same in the presence as well as in the absence of potassium ions. Therefore, cells with antibiotics induced channels remain

Table 4 The comparison of colony forming units values determined for *S. cerevisiae* standard strain (AD1234568) in conditions of the energy transfer measurements

Strain: AD1234568	AmB [μM]	Viable cells (% of control)
0.9% NaCl	2	54.5
	5	18.6
0.1 M KCl	2	100
	5	70.4

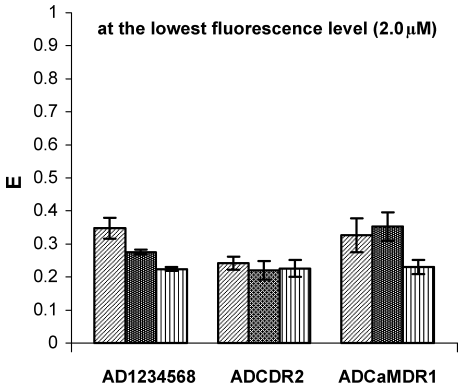


Fig. 2 Energy transfer efficiency from TMA-DPH to AmB and derivatives in *S. cerevisiae* standard strain and its MDR mutants.

Antibiotic concentration in the medium was 2.0 μM. ▨ AmB, ▩ AMA, ▥ MFAME.

viable. In contrast, when cells are suspended in saline, permeability changes induced by antibiotic led to replacement of the intracellular potassium by external proton and sodium ions with subsequent cell death due to the metabolic destabilization.

The profiles of the fluorescence intensity changes induced by antibiotics were mono- or biphasic depending on antibiotic concentration as well as on fungal strain tested.

Biphasic profile was observed for 2.0 and 5.0 μM concentrations of AmB, AMA and MFAME in standard strain and ADCR2 transformant. In ADCaMDR1 monophasic profile was observed for 2.0 and 5.0 μM concentrations of AmB, 2.0 μM AMA and MFAME whereas biphasic for 5.0 μM concentrations of AMA and MFAME.

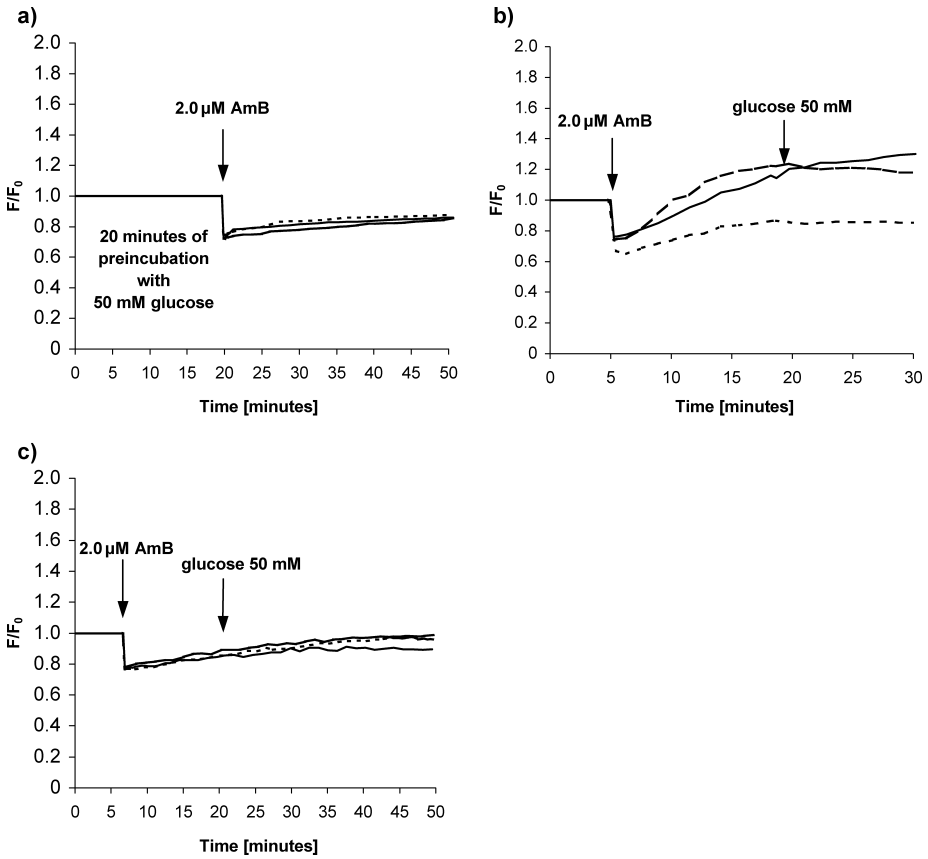


Fig. 3 Time courses of TMA-DPH fluorescence intensity in *S. cerevisiae* standard strain and its MDR mutants in the presence of AmB added to: a) cells washed and resuspended in fresh 0.1 M KCl after 20 minutes preincubation with 50 mM glucose b) cells suspended in 0.1 M KCl 5 minutes after TMA-DPH addition c) cells washed and resuspended in fresh 0.1 M KCl after 20 minutes preincubation with TMA-DPH.

F_0: Initial fluorescence intensity of TMA-DPH incorporated into membrane in the absence of antibiotic. F: Fluorescence intensity of TMA-DPH after antibiotic addition. —— AD1234568, —--— ADCDR2, - - - - ADCaMDR1.

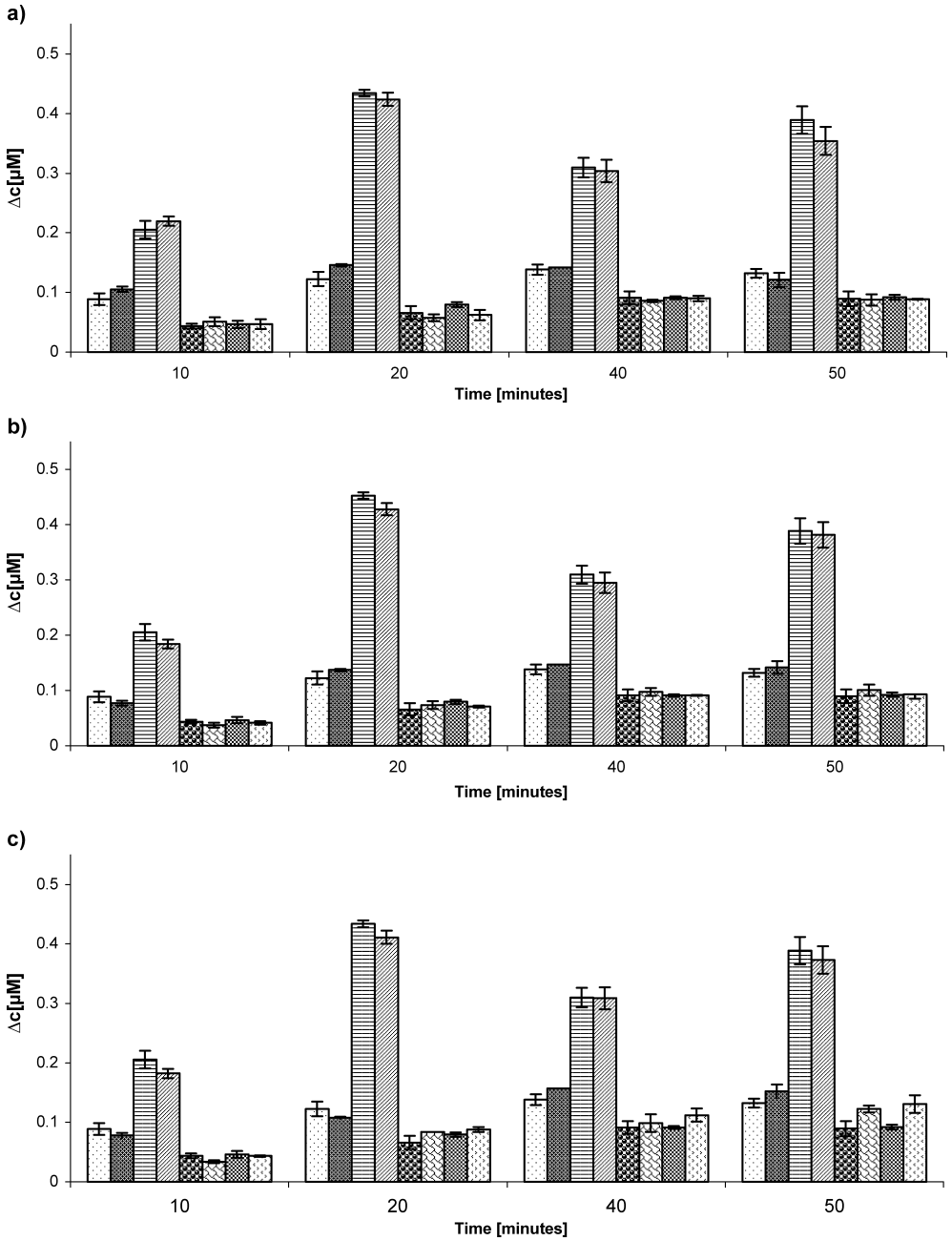


Fig. 4 R6G glucose-induced efflux from AD1234568 standard and its ADCDR2 mutant cells with or without treatment with 2 μM a) AmB, b) AMA, c) MFAME.

R6G efflux is expressed as a Δc: Total change of extracellular dye concentration calculated in relation to its initial level determined at time zero (immediately after addition of glucose). a) ADCDR2 without glucose, ADCDR2 AmB with glucose, AD1234568 with glucose, ADCDR2 AmB without glucose, AD1234568 without glucose, AD1234568 AmB with glucose, ADCDR2 with glucose, AD1234568 AmB without glucose. b) ADCDR2 without glucose, ADCDR2 with glucose, AD1234568 without glucose, AD1234568 with glucose, ADCDR2 AMA without glucose, ADCDR2 AMA with glucose, AD1234568 AMA without glucose, AD1234568 AMA with glucose. c) ADCDR2 without glucose, ADCDR2 with glucose, AD1234568 without glucose, AD1234568 with glucose, ADCDR2 MFAME without glucose, ADCDR2 MFAME with glucose, AD1234568 MFAME without glucose, AD1234568 MFAME with glucose.

Energy transfer efficiencies (E) calculated at the highest quenching have similar values for AmB, AMA and MFAME in three strains studied (Fig. 2). It suggests that AmB and the derivatives have similar membrane affinity and stability in standard strain and its MDR transformants.

Experiments with metabolizing cells enabling the supply of metabolic energy for extruding pumps in MDR cells have shown that neither membrane probe nor antibiotics are extruded from the membranes of standard and transformant strains during incubation with glucose (Fig. 3a). Extent of the fluorescence quenching as well as the fluorescence profiles were not affected by the presence of glucose in none of the strains studied. Time courses of the fluorescence intensity changes induced by AmB (Fig. 3a~c) and derivatives (data not shown) were practically the same. Stable binding of the antibiotics with cells was confirmed by experiments in which cells containing fluorescent probe and the antibiotic were separated and then transferred to fresh medium. For all strains and all compounds studied fluorescence intensity remained unchanged in the presence as well as in the absence of glucose (data not shown).

Lack of marked differences in cell affinities as well as of antifungal and permeabilizing activities of AmB, AMA and MFAME to the resting and metabolizing strains studied suggested that none of these antibiotics is substrate of the extrusion pumps neither belonging to ABC nor MFS superfamilies providing that the examined compounds do not affect pumps expression or functioning.

R6G-Loaded Cells

In order to verify the assumption that AmB and its derivatives are able to overcome fungal MDR by not being the substrates of the multidrug exporting pumps and do not affect their efficiency we performed experiments with fungal cells labeled with R6G. This cationic fluorescent dye is known to be transported by a wide variety of MDR proteins in yeast as well as in mammalian cells [36]. As a substrate for several ABC transporters, including Cdr1p and Cdr2p, R6G was used earlier to monitor their drug efflux activity [29, 32, 37].

In our studies we compared the ability of parental strain AD1234568 and its MDR transformant-ADCDR2 to R6G efflux in the absence and in the presence of a source of energy (glucose) and examined the effects of AmB and its derivatives on this process.

The level of R6G uptake in the absence of glucose was the same for AD1234568 parent strain (8.92 ± 0.028 nmol/ml) and ADCDR2 transformant (8.60 ± 0.060 nmol/ml). Both strains accumulated equivalent amounts of R6G, which entered into fungal cells by passive diffusion.

The energy dependent extrusion of R6G into the extracellular medium was observed only for MDR transformant in the presence of glucose. Results presented in Fig. 4a~c indicate that neither AmB nor its derivatives change this pattern. The data evidence that in the presence of examined antibiotics extrusion pumps are still expressed and functioning. Taking into consideration results obtained with membrane probe and R6G we can conclude that out of five possible mechanisms of overcoming MDR mentioned in the Introduction, the antibiotics studied exhibit activity towards MDR strains because they are not substrates for drug exporting pumps.

Molecular Volumes of Antibiotics

Looking for explanation of reasons why examined antibiotics appeared to be non-substrates of MDR pumps we thought reasonable to draw the attention to the experimental evidence, which indicate that interaction with MDR transporters is strongly dependent on the molecular size of the substrate [10]. This factor is thought to be critical and independent of any requirement for hydrophobicity. It was found that Pdr5p substrates have surface volumes greater than 90 $Å^3$ with an optimum response at 200~225 $Å^3$. Further increase of molecular volume has negative effect on transporter driven efflux.

Using the procedure described in the Material and Methods section we calculated the molecular volumes of AmB, AMA and MFAME to be 985, 988 and 1178 $Å^3$, respectively. Considering the surface volumes of AmB and its derivatives it can be presumed that these compounds are too large to fulfill the steric requirement of MDR pumps to be their substrates.

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