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Unusual Susceptibility of a Multidrug-Resistant Yeast Strain to Peptidic Antifungals

SŁAWOMIR MILEWSKI,[1]* FIORENZO MIGNINI,[2] RAJENDRA PRASAD,[3] AND EDWARD BOROWSKI[1]

Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, Gdańsk, Poland[1]; Department of Cellular, Molecular and Animal Biology, University of Camerino, Camerino, Italy[2]; and School of Life Sciences, Jawaharlal Nehru University, New Delhi, India[3]

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The susceptibility of *Saccharomyces cerevisiae* JG436 multidrug transporter deletion mutant, Δ*pdr5*, to several antifungal agents was compared to that of JG436-derived JGCDR1 and JGCaMDR1 transformants, harboring the *CDR1* and *CaMDR1* genes, encoding the main drug-extruding membrane proteins of *Candida albicans*. The JGCDR1 and JGCaMDR1 yeasts demonstrated markedly diminished susceptibility to the azole antifungals, terbinafine and cycloheximide, while that to amphotericin B was unchanged. Surprisingly, JGCDR1 but not JGCaMDR1 cells showed enhanced susceptibility to peptidic antifungals, rationally designed compounds containing inhibitors of glucosamine-6-phosphate synthase. It was found that these antifungal oligopeptides, as well as model oligopeptides built of proteinogenic amino acids, were not effluxed from JGCDR1 cells. Moreover, they were taken up by these cells at rates two to three times higher than by JG436. The tested oligopeptides were rapidly cleaved to constitutive amino acids by cytoplasmic peptidases. Studies on the mechanism of the observed phenomenon suggested that an additive proton motive force generated by Cdr1p stimulated uptake of oligopeptides into JGCDR1 cells, thus giving rise to the higher antifungal activity of FMDP [N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid]-peptides.

The phenomenon of multidrug resistance is considered to be the major challenge for modern chemotherapy. Although better recognized and characterized in the case of cancer and bacterial cells, it is also becoming an emerging problem in antifungal chemotherapy, thus making worse the already difficult situation resulting from an increasing number of immunocompromised patients, the appearance of new human pathogenic fungi, and the very limited number of available antifungal chemotherapeutic agents (11). The molecular mechanism underlying the multidrug resistance is an overexpression of membrane proteins belonging to different classes of energy-dependent efflux pumps. Most of these proteins are the members of a family of ATP-binding cassette (ABC) transporters. A number of them have been already identified in fungi, including Cdr1p and Cdr2p in *Candida albicans* (30, 32) and Pdr5p in *Saccharomyces cerevisiae* (2). Moreover, transporters of another type, the major facilitator superfamily (MFS), have been also detected, including a product of the *CaMDR1* gene in *C. albicans* (6). The substrate spectrum of fungal multidrug transporters covers most of the drugs used in clinics for the treatment of disseminated infections, including fluconazole and itraconazole (1). On the other hand, the multidrug-resistant fungi retain in most cases the susceptibility to membrane-affecting agents: amphotericin B (31) and basic oligopeptides (13). This is in agreement with a general rule assuming that the "classical" substrate for drug-extruding pumps is a predominantly hydrophobic molecule, usually bearing a localized positive or negative charge, penetrating the cell membrane by free diffusion (36). However, some intracellularly acting antifungal compounds are transported into the cells by active transport systems. This group includes a known drug, 5-fluorocytosine (8), and several antifungal agents that have not reached clinics so far, including peptidic compounds, containing FMDP [N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid], an inhibitor of glucosamine-6-phosphate (GlcN-6-P) synthase (24). Any data on the affinity of these compounds to multidrug efflux pumps have not been reported yet.

We present here the results of our recent studies on antifungal activity of FMDP-peptides against multidrug-resistant and drug-sensitive yeasts.

MATERIALS AND METHODS

Antifungal agents and other chemicals. Cycloheximide was from Sigma, St. Louis, Mo. Fluconazole and tioconazole were from Pfizer, Milano, Italy. Amphotericin B was from Bristol-Myers Squibb, New York, N.Y., and terbinafine was from Sandoz, Ltd., Basel, Switzerland. FMDP, Nva-FMDP, and Lys-Nva-FMDP were synthesized by R. Andruszkiewicz, Technical University of Gdańsk. All other chemicals were from Sigma.

Yeast strains and growth conditions. *S. cerevisiae* ATCC 9763 cells were stored on Sabouraud agar slants and propagated in Sabouraud liquid medium at 30°C with shaking. *S. cerevisiae* JG436 (MAT**a** *pdr5*::Tn*5 leu2 met5 ura3-52 mak71 KRB1*) was a kind gift from J. Golin, Catholic University of America, Washington, D.C. This strain was hypersensitive to cycloheximide and some other unrelated antifungal agents (20). JG436 was transformed with *CaMDR1*-carrying plasmid pNC39 and *CDR1*-carrying plasmid pS12, which had a common vector background of pYEUra3 (12, 30). The resulting JG436 transformants are designated as JGCaMDR1 and JGCDR1. The JG436 yeast cells were propagated in a yeast nitrogen base-glucose (YNBG) minimal medium containing 0.67% YNB without amino acids (Difco), 2% glucose, L-methionine at 20 μg ml^{-1}, L-leucine at 40 μg ml^{-1}, and uracil at 30 μg ml^{-1}, while the transformant strains were maintained in a similar medium but lacking uracil.

Antifungal susceptibility tests. MICs were determined by the serial twofold dilution method in 96-well microtiter plates in the minimal YNBG medium described above for the JG436 strain. The inoculum size was 10^4 cells ml^{-1}. Plates were incubated for 24 h at 30°C. The MIC was defined as the lowest drug concentration preventing visible growth. Each compound was tested at least three times. Determination of the pH dependence of the MIC was performed in

* Corresponding author. Mailing address: Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, 11/12 Narutowicza St., 80-952 Gdańsk, Poland. Phone: 48-58-3472107. Fax: 48-58-3472694. E-mail: milewski@altis.chem.pg.gda.pl.

RPMI 1640 medium with glutamine, without sodium bicarbonate but containing 2% glucose, buffered with 0.165 M MOPS (morpholinepropanesulfonic acid), with the pH adjusted to the appropriate level with 1 M HCl or 1 M NaOH (26). The inoculum size, incubation conditions, and MIC definition were the same as those described above.

Determination of peptide uptake rates. Yeast cells grown exponentially in YNBG medium were harvested by centrifugation (3,000 × *g*, 5 min), washed with 50 mM potassium phosphate buffer (pH 5.0 or 6.5), and suspended in the same buffer containing 1% glucose to a final cell density corresponding to an A_{660} of 1.0. The cell suspension was incubated at 30°C. After 10 min, an oligopeptide solution was added to give a final concentration of 100 μM. At that moment and at 5-min intervals thereafter, 2-ml samples of the cell suspension were withdrawn and immediately filtered through the Whatman GF/A filters, and the filtrates were used to determine the residual peptide concentration. Then, 1-ml portions of the filtrates were taken and combined with 1.25-ml aliquots of a solution containing 4% $Na_2B_4O_7 \cdot 10H_2O$ and 0.8 mg of 2,4,6-trinitrobenzenesulfonate (TNBS) ml^{-1}. The reaction was carried out at 37°C for 30 min. The A_{420} value was measured, and the peptide concentration was read from the appropriate standard curve. Data were plotted as nanomoles of oligopeptide taken up by 1 mg (dry weight) of cells versus time. The initial uptake velocities were determined from the slopes of the linear part of the curves, in the 0- to 10-min region.

TLC. Qualitative analysis of the spent medium used for the determination of peptide uptake rate was performed by thin-layer chromatography (TLC). Small aliquots of filtrates were applied to silica gel-coated aluminum sheets. Chromatograms were developed in two solvent systems: system A, *n*-butanol–acetic acid–water (4:1:1), and system B, chloroform-ethanol (2:1). The amino acids and peptides were visualized by ninhydrin staining and FMDP-containing compounds by quenching of UV light.

Preparation of cell extract. Yeast cells from the overnight culture on YNBG were harvested by centrifugation and washed with cold 25 mM potassium phosphate buffer (pH 6.5). Cells were then suspended in a minimal amount of the buffer and disrupted with French press. The resulting suspension was centrifuged (35,000 × *g*, 4°C, 45 min), and the supernatant was used as a cell extract for the determination of peptide cleavage rates.

Determination of peptide cleavage rates. The incubation mixtures, consisting of 10 ml of a 200 μM peptide solution in 50 mM potassium phosphate buffer (pH 6.5) and 2 ml of appropriately diluted crude extract (final protein concentration, 0.1 to 0.5 mg ml^{-1}), were incubated at 30°C. At 5-min intervals, 2-ml aliquots were withdrawn and heated at 100°C for 3 min. The resulting suspensions were centrifuged to remove protein precipitates, and the concentration of free amino acids in the supernatant was determined by the Cd-ninhydrin procedure (9).

Determination of initial velocity of proton efflux. The initial velocities of proton extrusion by mutant yeast cells were determined according to the procedure described previously (14). Briefly, yeast mutant cells grown in the YNBG medium lacking uracil were harvested in the mid-exponential phase of growth, washed twice with water, and suspended in fresh, unbuffered water at 10^8 cells ml^{-1}. Cell suspensions were preincubated for 5 min at 30°C in a water-circulating chamber with constant stirring. The incubation was continued, and the pH of the cell suspensions was monitored with a PHM-62 pH meter (Radiometer, Copenhagen, Denmark). The release of protons was recorded for 5 min. The corresponding amount of proton released was then calculated after we corrected for the buffer capacity of the yeast suspension, as determined by the addition of a known amount of 10 mM HCl solution.

Other methods. The protein concentration was measured by the Bradford procedure (7), using bovine serum albumin as a standard. The activity of GlcN-6-P synthase was assayed in cell extracts according to a previously published method (18). One unit of specific activity was defined as an amount of enzyme that catalyzed formation of 1 μmol of GlcN-6-P h^{-1} mg of $protein^{-1}$.

RESULTS AND DISCUSSION

Genetically modified yeast mutant cells, JG436, lacking the main yeast drug extruding pump Pdr5p and its transformants containing *C. albicans* genes encoding Cdr1p and CaMdr1p drug exporters, respectively, can serve as useful research tools for studies on substrate specificity of candidal drug efflux systems. We used these transformants for the determination of in vitro growth inhibitory activity of several antifungal agents. The results of this experiment are shown in Table 1. As could be expected, both JGCDR1 and JGCaMDR1 cells demonstrated reduced susceptibility to a number of antifungals compared to the parent JG436 strain, while the activity of the membrane-active antifungal compound amphotericin B was exactly the same against all types of mutant cells. On the other hand, JGCDR1 yeast cells demonstrated enhanced susceptibility to Nva-FMDP and Lys-Nva-FMDP, whereas JGCaMDR1 was unchanged compared to JG436. A similar phenomenon was also observed for other FMDP-oligopeptides, containing two to four amino acid residues (data not shown). The susceptibility of JG436 cells to antifungal agents under study was the same or slightly higher than that of the standard yeast strain ATCC 9763. The MIC values for FMDP-peptides against yeast transformants were two to four times lower, when the determination was made in YNBG medium containing L-glutamate at 2 mg ml^{-1}, instead of ammonium sulfate as a nitrogen source (data not shown). This difference probably reflects the nitrogen catabolite repression of peptide transport systems, which is well known and characterized in *S. cerevisiae* (5). Moreover, it should be mentioned that FMDP-peptides are generally much more active against human pathogenic fungi, especially *C. albicans* (23, 24), than against baker's yeast.

TABLE 1. Growth-inhibitory activity of several antifungal agents against yeast mutants and yeast standard strain[a]

Antifungal agent	MIC[b] (μg ml^{-1}) against strain:			
	JG436	JGCDR1	JGCaMDR1	ATCC 9763
Fluconazole	3.2	25	50	6.4
Tioconazole	0.1	1.6	1.6	0.1
Terbinafine	8	32	16	8
Amphotericin B	0.2	0.2	0.2	0.2
Cycloheximide	0.05	0.8	1.6	0.2
Nva-FMDP	25	3.2	25	25
Lys-Nva-FMDP	200	25	200	200

[a] MICs were determined by the microtiter serial twofold dilution method in YNB medium containing 1% glucose. Inoculum size was 10^4 cells ml^{-1}. Plates were incubated for 24 h at 30°C.

[b] MIC was defined as the lowest concentration of an antifungal agent preventing visible growth.

It was previously shown that FMDP-peptides are transported into *C. albicans* cells by peptide permeases and cleaved intracellularly by peptidases, and the released FMDP inhibits activity of the enzyme L-glutamine:D-fructose 6-phosphate amidotransferase (EC 2.6.1.16, known as GlcN-6-P synthase) (24). In consequence, the biosynthesis of the glucosamine-containing cell wall macromolecules, chitin and mannoprotein, is inhibited (24). It was also evidenced that the relative rates of uptake of an oligopeptide antifungal agents determine their anticandidal activity (22). Therefore, trying to find an explanation for unusual susceptibility of JGCDR1 yeast to FMDP-peptides, we determined the uptake rates of these compounds and a few oligopeptides built exclusively of proteinogenic amino acids by JG436 transformants cells. Determinations of uptake rates of small molecules into microbial cells are usually made using radiolabeled compounds. However, it is known that in the case of oligopeptides this approach may result in false results due to the efflux of degradation products containing radioisotopes (28). We used our own method based on the colorimetric assay of yellow products of reaction between peptides and TNBS (23). This approach is based upon the assumption that a peptide is the only compound reacting with TNBS that is present in the external medium. Therefore, our exper-

TABLE 2. Initial velocities of oligopeptide uptake by yeast mutants

Peptide	External pH	Mean initial uptake rate[a] (nmol min^{-1} mg [dry wt]$^{-1}$) ± SD by:		
		JG436	JGCDR1	JGCaMDR1
Nva-FMDP	5.0	1.81 ± 0.22	3.03 ± 0.33	1.75 ± 0.14
	6.5	0.94 ± 0.16	1.82 ± 0.22	0.91 ± 0.15
Lys-Nva-FMDP	5.0	1.11 ± 0.12	1.95 ± 0.17	1.01 ± 0.15
	6.5	0.45 ± 0.15	0.87 ± 0.08	0.49 ± 0.08
Leu-Met	5.0	4.87 ± 0.28	5.84 ± 0.42	4.66 ± 0.48
	6.5	3.20 ± 0.31	4.15 ± 0.38	3.12 ± 0.28
His-Met	5.0	5.25 ± 0.44	5.68 ± 0.29	5.08 ± 0.33
	6.5	4.30 ± 0.25	4.88 ± 0.52	4.30 ± 0.30
Met-Met-Met	5.0	2.81 ± 0.11	3.64 ± 0.32	2.62 ± 0.22
	6.5	1.17 ± 0.09	1.94 ± 0.16	1.31 ± 0.11
Leu-Leu-Leu	5.0	3.25 ± 0.06	5.02 ± 0.44	3.22 ± 0.40
	6.5	1.85 ± 0.30	4.45 ± 0.32	1.91 ± 0.21
$(Ala)_5$	5.0	1.42 ± 0.10	2.73 ± 0.18	1.50 ± 0.15
	6.5	0.22 ± 0.04	1.63 ± 0.17	0.33 ± 0.05

[a] Suspensions of yeast cells in 50 mM potassium phosphate buffer (pH 5.0 or 6.5) containing 1% glucose (final cell density corresponding to $A_{660} = 0.8 \div 1.0$) were incubated at 30°C. Samples (2 ml) of the cell suspensions were withdrawn immediately after the addition of an oligopeptide (100 μM) and at 5-min intervals thereafter. Cells were removed by filtration, and the concentrations of the oligopeptides remaining in the filtrates were determined by the TNBS method. Values are the means of five determinations ± the standard deviation.

iment was carried out in a phosphate-buffered glucose solution, and no amino group-containing compounds were added except the tested oligopeptide. The examined peptides were continuously taken up by all tested types of yeast mutants. The uptake was linear for at least 15 to 20 min, and then its velocity gradually decreased, thus reflecting the influence of decreasing concentration of the oligopeptide in the medium. The initial uptake velocities of several oligopeptides by JG436 and JGCDR1 cells, determined from the slopes of the linear part of the experimental curves, are demonstrated in Table 2. The results show that all tested oligopeptides were taken up by *CDR1*-expressing JGCDR1 cells at higher rates than by JG436 yeast lacking Cdr1p. On the other hand, the oligopeptide uptake by both yeast transformants was faster at pH 5.0 than at pH 6.5. This finding is consistent with the previous report on properties of yeast oligopeptide transport systems (4). It is also noteworthy that the differences between the uptake rates exhibited by JGCDR1 and JG436 were in most cases larger at pH 6.5 than at pH 5.0. The initial velocities of oligopeptide uptake by JGCaMDR1 cells were almost identical at both pH values to those demonstrated by JG436.

FMDP was very slowly taken up by yeast mutants. The initial velocities of its uptake were within the range of 0.1 to 0.3 nmol min^{-1} mg (dry weight)$^{-1}$.

The TLC analysis revealed that the tested oligopeptides were the only ninhydrin-positive compounds present in the spent media. No spots corresponding to the constitutive amino acids were detected. We did not also detect any UV light-absorbing compounds in the spent medium separated from cells treated with Nva-FMDP or Lys-Nva-FMDP, except the FMDP-oligopeptides themselves. Nevertheless, some especially fast-absorbed oligopeptides (e.g., His-Met or Leu-Leu-Leu) were completely removed from the medium after 20 to 30 min, when no compound reacting with TNBS could be detected any longer. The same was true after 40 to 50 min for FMDP-peptides, which were taken up at lower rates. Moreover, the shapes of all experimental curves obtained by us in uptake experiments were similar, a result characteristic for the continuous inward transport by permease, and not affected by any efflux phenomena. We can therefore conclude that neither the tested oligopeptides nor their constitutive amino acids were effluxed from the yeast JG436 and its *CDR1* and *CaMDR1* transformants.

It was previously shown that the effectiveness of anticandidal action of FMDP-peptides is influenced by three factors: the velocity of uptake, the rate of intracellular cleavage, and the affinity for the intracellular target, with the first factor being crucial for the overall activity (24). We therefore determined the rates of cleavage of Nva-FMDP and Lys-Nva-FMDP by peptidases present in cell extracts prepared from mutant yeast. The results of this experiment shown in Table 3 clearly demonstrate that the rates were practically the same, irrespectively of the source of the cell extract used for determination. On the other hand, the cleavage rates were much higher than the corresponding uptake velocities shown in Table 2. The TLC analysis of solutions containing FMDP-peptides (100 μM) incubated at 30°C in the presence of crude extracts (protein concentration, 1 mg ml^{-1}) confirmed a very quick release of free amino acids. Spots corresponding to FMDP appeared as quickly as within 45 to 60 s. One may doubt whether the results of this in vitro experiment can be related to the intracellular conditions. However, a very fast cleavage of different oligopeptides internalized by yeast cells was previously demonstrated (4, 17), and a correlation between results obtained under in vitro and in vivo conditions was also shown (25).

There was no difference in the specific activity of GlcN-6-P synthase present in cell extracts prepared from yeast mutants and the sensitivity of this enzyme to inhibition by FMDP. The specific activity of the enzyme was 0.046 ± 0.003 U, and the 50% inhibitory concentration for FMDP was 7.5 ± 0.2 μM. It is therefore clear that neither the rate of enzymatic hydrolysis nor the interaction of FMDP with its intracellular target influence the growth-inhibitory activity of FMDP-peptides against yeast mutants. This seems to be exclusively determined by the inward transport rates. A similar conclusion was previously drawn for the anticandidal activity of FMDP-peptides (23) and some other peptidic antifungals (22).

Since we observed that the FMDP-peptide uptake rate was pH dependent (Table 2), it seemed reasonable to establish the pH dependence of the antiyeast activity of FMDP-peptides. This was done by determining the MICs in buffered RPMI media. The medium composition and conditions of this assay were in compliance with recommendations of the National

TABLE 3. Rates of cleavage of FMDP-peptides by peptidases present in crude extracts prepared from yeast mutant cells

FMDP-peptide	Mean cleavage rate[a] (nmol min^{-1} mg of protein^{-1}) ± SD		
	JG436	JGCDR1	JGCaMDR1
Nva-FMDP	13.7 ± 2.2	14.4 ± 2.8	15.2 ± 0.9
Lys-Nva-FMDP	18.1 ± 1.6	16.2 ± 1.3	18.0 ± 2.0

[a] Mixtures containing 200 μM peptide solution in 50 mM potassium phosphate buffer (pH 6.5) and appropriately diluted crude extract, prepared from exponentially growing yeast cells, were incubated at 30°C. Samples of incubation mixtures were collected at 5-min intervals and deproteinized, and the concentration of free amino acids in the supernatant left after removal of protein precipitate was determined by the Cd-ninhydrin procedure. Values are the means of three independent determinations ± the standard deviation.

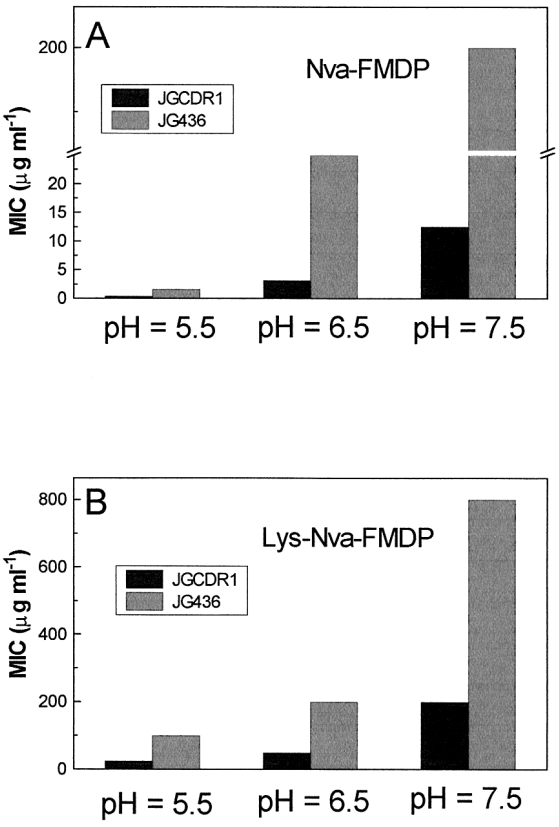

FIG. 1. pH dependence of antifungal in vitro activity of Nva-FMDP (A) and Lys-Nva-FMDP (B) against JG436 and JGCRD1 cells. MICs were determined by microtiter serial twofold dilution method in RPMI medium buffered with 0.165 M MOPS. Inoculum size was 10^4 cells ml^{-1}. Plates were incubated for 24 h at 30°C, and the results were read visually.

Committee for Clinical Laboratory Standards (26). The results (Fig. 1) clearly demonstrate that the growth-inhibitory activity of FMDP-peptides against both JG436 and JGCDR1 cells was strongly enhanced in acidic media. A similar phenomenon was also observed for JGCaMDR1 (data not shown).

Oligopeptides containing two to six amino acid residues are transported into *C. albicans* and *S. cerevisiae* cells by energy-dependent permeases. The extensive studies on peptide transport system in *C. albicans* led to the identification and characterization of at least two components: the di- and tripeptide permease and the oligopeptide permease transporting tri-, tetra-, penta-, and hexapeptides (3, 5, 21, 23). In *S. cerevisiae*, a product of the *PTR2* gene was unequivocally identified as a di- and tripeptide permease, with very low affinity for longer oligopeptides (29). The molecular mechanism of peptide translocation by Ptr2p is not known, but the pH dependence of its activity, with a pH optimum of 5.5 (16) and a sequence homology to other members of the PTR family of transport proteins (35), indicate that this permease acts as an H^+-oligopeptide symporter. We were able to confirm this assumption by demonstrating the strong inhibitory effect of the known proton shuttle, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on peptide uptake by yeast mutants and their transformants (Fig. 2). The presence of this agent (100 μM) practically completely stopped the oligopeptide uptake.

The only difference between JG436 and JGCDR1 cells is the presence of the Cdr1p efflux pump in the latter. It is therefore clear that the unusually high susceptibility of the JGCDR1 cells to FMDP-peptides should be a consequence of a possible activity of this ABC transporter. One of the theoretical suggestions for the mechanism of this phenomenon could be the action of Cdr1p as an additional oligopeptide permease. This idea seemed very unlikely since this protein is known to act exclusively as an export pump, but experimental verification was required. Such evidence was provided by the results of the experiment in which the growth-inhibitory activity of FMDP-peptides and cycloheximide was determined in the presence of the calcium channel blocker, verapamil, which is known to be a very good substrate for P-glycoprotein (37). The data presented in Table 4 show that the presence of this compound strongly increases the susceptibility of JGCDR1 cells to the action of cycloheximide and has no effect on susceptibility of JG436 yeast. This result was expected since Cdr1p blocked by verapamil was supposed to be switched off as a potential way of cycloheximide efflux. On the other hand, verapamil had no

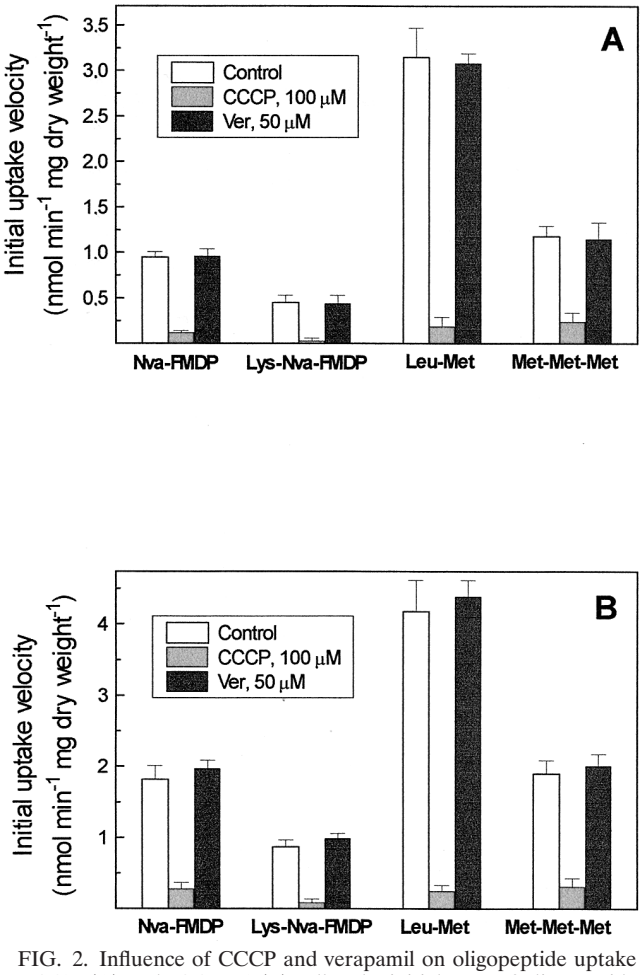

FIG. 2. Influence of CCCP and verapamil on oligopeptide uptake by JG436 (A) and JGCDR1 (B) cells. The initial rates of oligopeptide uptake were determined in 50 mM phosphate buffer (pH 6.5) containing 1% glucose. Other details were the same as those described in the legend to Table 2. Each bar represents the mean of three independent determinations ± the standard deviation.

TABLE 4. Influence of verapamil on antifungal in vitro activity of FMDP-peptides and cycloheximide against JG436 and JGCDR1 cells[a]

Antifungal agent	Verapamil concn (μM)	MIC (μg ml^{-1}) against:	
		JG436	JGCDR1
Nva-FMDP	0	25	3.12
	50	25	6.25
Lys-Nva-FMDP	0	200	25
	50	200	25
Cycloheximide	0	0.05	0.8
	50	0.05	0.1

[a] Conditions of the determination were as described in the legend to Table 1.

effect on the antifungal activity of FMDP-peptides against JG436 and JGCDR1. No effect was also observed in the case of JGCaMDR1 cells (data not shown). We did not note any substantial influence of verapamil on oligopeptide uptake to yeast transformants, except the very slight stimulation of their uptake in the case of JGCDR1 cells (Fig. 2). Any competition between FMDP-peptides and verapamil for Cdr1p can therefore be excluded.

Another possibility which may be taken into account as a probable mechanism of observed supersensitivity is the creation of an additional driving force for peptide transport due to the presence of Cdr1p. This protein is a *C. albicans* homologue of the human Mdr1p efflux pump (30), well known as a P-glycoprotein. This is therefore likely that the substrate specificity of both drug exporters should be similar. In this respect, it is worth mentioning that P-glycoprotein is known to export some peptides. However, we must stress that this opinion concerns some very specific compounds, e.g., ionophoric peptides such as gramicidin and valinomycin or hydrophobic cyclic and linear modified peptides or peptide derivatives such as NAc-L-leucyl-L-leucyl-L-methioninal, pepstatin A, cyclosporin A, or leupeptin (33, 34). Ionophoric peptides were also shown to be the substrates for yeast Pdr5p (19). Small, unmodified linear peptides have never been studied in this respect. On the other hand, Fritz et al. reported quite recently that human Mdr1p (P-glycoprotein) overexpressed in *S. cerevisiae*, acts at low external pH as a H^+ efflux and Cl^- influx pump, whereas at a pH of >8 it promotes ion translocation in the opposite directions (10). It was even suggested that biophysical perturbations due to abnormal ion transport might be a natural activity of this protein and the multidrug resistance phenotype could be fully explained by changes in the membrane potential and proton gradient generated by human Mdr1p (15). The structural homology between Cdr1p and human P-glycoprotein allows us to put forward a working hypothesis that Cdr1p expressed in *S. cerevisiae* may also create an additional proton motive force. This should in turn drive the oligopeptide transport by an H^+-dependent peptide permease and thus enhance the antifungal activity of FMDP-peptides. In order to verify this hypothesis we determined the initial velocities of proton efflux from yeast mutant cells suspended in unbuffered water. The results of this experiment (Fig. 3) clearly demonstrate that the JGCDR1 cells effluxed almost three times more protons per minute than did the JG436 and JGCaMDR1 cells. Our hypothesis has been additionally strengthened by an identical growth-inhibitory activity of FMDP-peptides against JG436 and JGCaMDR1 and similar uptake rates of oligopeptides by both transformant cells. The drug efflux pump CaMdr1p present in JGCaMDR1 belongs to the MFS of drug exporters. The MFS proteins act as H^+-substrate antiporters (27), so that their action cannot create any additional proton motive force. Since, on the other hand, our data show that CaMdr1p extrudes neither FMDP-peptides nor their constitutive amino acids, it is not surprising that the presence of this pump has no effect on antifungal activity of FMDP-peptides.

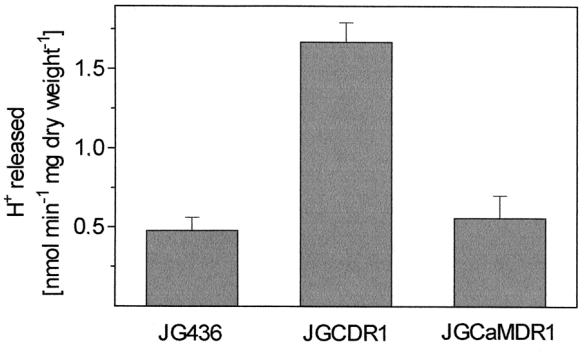

FIG. 3. Initial velocities of proton efflux by yeast mutants. The initial rates of proton efflux were determined by monitoring the pH changes of yeast cell suspensions. Yeast mutant cells were transferred from the minimal growth medium to unbuffered water, and pH changes were recorded. Each bar represents the mean of three independent determinations ± the standard deviation.

To our best knowledge, FMDP-peptides are the first reported example of antimicrobial agents that are more active against multidrug-resistant cells. Although the hypothesis on the molecular mechanism of this supersensitivity needs further experimental evidence, the phenomenon itself seems to open the new possibilities of overcoming the multidrug resistance problem. In our opinion this could be done by the application of structural mimics of natural metabolites: peptides, amino acids, or sugars as antimicrobial agents. Such compounds are transported unidirectionally into the cells by respective permeases and are less likely to be extruded by drug efflux pumps than xenobiotics. Moreover, our results presented above indicate that, at least in some cases, i.e., agents transported into the cells by H^+-substrate symporters, there is a chance for the paradoxically enhanced susceptibility of potentially "resistant" cells. Work is in progress in our laboratory to verify this hypothesis.

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