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Design of novel peptide analogs with potent fungicidal activity, based on PMAP-23 antimicrobial peptide isolated from porcine myeloid

Dong Gun Lee,[a] Pyung Il Kim,[a] Yoonkyung Park,[a] Eun-Rhan Woo,[a] Ji Suk Choi,[a] Cheol-Hee Choi,[a,b] and Kyung-Soo Hahm[a,*]

[a] *Research Center for Proteineous Materials (RCPM), Chosun University, 375 Seosuk-Dong, Dong-Ku, Kwangju 501-759, Republic of Korea*
[b] *Department of Pharmacology, Chosun University Medical School, 375 Seosuk-Dong, Dong-Ku, Kwangju 501-759, Republic of Korea*

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Abstract

PMAP-23 is a 23-mer peptide derived from porcine myeloid. To develop novel antifungal peptides useful as therapeutic drugs, it would require a strong fungicidal activity against pathogenic fungal cells. To this goal, several analogs, with amino acid substitutions, were designed to increase the net hydrophobicity by Trp (W)-substitution at positions 10, 13, or 14 at the hydrophilic face of PMAP-23 without changing the hydrophobic helical face. The Trp (W)-substitution (P6) showed an enhanced fungicidal and antitumor activities, with the fungicidal activity inhibited by salts and the respiratory inhibitor, NaN_3. The results suggested that the increase of hydrophobicity of the peptides correlated with fungicidal activity. The fungicidal effects of analog peptides were further investigated using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a membrane probe. In *Candida albicans*, the analog peptide (P6) exerted its fungicidal effect on the blastoconidia in 20% fetal bovine serum by disrupting the mycelial forms. Furthermore, P6 caused significant morphological changes, and these facts suggested that the fungicidal function of the novel analog peptide (P6) was by damaging the fungal cell membranes. Thus, this peptide may provide a useful template for designing novel antifungal peptides useful for the treatment of infectious diseases.

Keywords: PMAP-23; Antifungal peptides; Analog peptides; Hydrophobicity

Antimicrobial peptides have been recognized as playing important roles in the innate host defense mechanisms of most living organisms including plants, insects, amphibians, and mammals [1–6]. They are also known to possess potent antibiotic activity against bacteria, fungi, and even certain viruses [7–11].

Recently, the rapid emergence of microorganism pathogens, which are resistant to currently available antibiotics, has triggered considerable interest in the isolation and investigation of the mode of action of antimicrobial peptides.

Antibiotics act in two different ways, first by targeting the physiology of the pathogen, and second, by disrupting the cellular structure of the host cells. In the latter case, antibiotics are relatively safe from encounters with resistant host strains, since mutations in the overall plasma membrane structure are less likely than variations in the enzyme physiology within a host cell. Therefore, the disruption of plasma membranes, by pore or ion channel formation, has been widely used in antibiotic agents.

In mammals, the cytoplasmic granules of neutrophiles are an abundant source of a number of antimicrobial peptides. cDNAs of several neutrophil-derived antimicrobial peptides have been cloned. Among these, PMAP-23 peptide was identified by cDNA cloning, and has been shown to possess potent antibacterial activity with chemically synthesized peptides [12,13]. Also, PMAP-23 has already shown fungicidal effects and modes of action [14].

With this study, to obtain peptides having improved fungicidal activity, novel analog peptides with amino acid substitutions were designed and synthesized based on the sequence and α-helical wheel diagram of PMAP-23. In the present study, the fungicidal effect and anticancer activity of synthetic peptides, against pathogenic

* Corresponding author. Fax: +82-62-227-8345.
E-mail address: kshahm@mail.chosun.ac.kr (K.-S. Hahm).

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fungal and cancer cells, were measured. Additionally, we discussed the importance of PMAP-23 analogs, with respect to changes in plasma membrane dynamics, and their effectiveness in the dimorphic transition in *Candida albicans*, and damage caused to the fungal cell membrane.

Materials and methods

Peptide synthesis. The peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry [15]. Rink amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidate peptide. The coupling of Fmoc–amino acids was performed with *N*-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp), trityl (Gln), *tert*-butyloxycarbonyl (Lys). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethanedithiol, and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with diethyl ether, dried in vacuum, and purified using a reversed-phase preparative HPLC on a Waters' 15 μm Deltapak C_{18} column (19 × 30 cm). Purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, USA), 4.6 × 25 cm. The purified peptides were hydrolyzed with 6 N HCl at 110 °C for 22 h and then dried in vacuum. The residues were dissolved in 0.02 N HCl and subjected to an amino acid analyzer (Hitachi Model, 8500 A, Japan). Peptide concentration was determined by amino acid analysis. The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI mass spectrometer [16].

Fungal strains. Saccharomyces cerevisiae (KCTC 7296) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejon, Korea. *C. albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan.

Antifungal activity. The fungal strains were grown at 28 °C in potato dextrose broth (PDB) medium. The fungal cells were seeded in the wells of a 96-microtiter plate in PDB media at a density of 2×10^3) cells (100 μl per well). Ten μl of the serially diluted-peptide solution was added to each well and the cell suspension was incubated for 24 h at 28 °C. Ten μl of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution [5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well, and the plates were incubated further at 37 °C. Thirty μl of 20 % (w/v) SDS solution containing 0.02 M HCl was then added and the plates were incubated at 37 °C for 16 h to dissolve the formazan crystals that had formed [17,18]. The turbidity of each well was measured at 570 nm using a microtitrator ELISA reader (Molecular Devices Emax, CA, USA).

Anticancer activity. The in vitro cytotoxicity of peptides was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] assay. Cells were seeded at 2×10^5 cells/ml in α-MEM medium containing 10% FBS. Ninety μl aliquots of this suspension were seeded into a 96-well microplate containing already 10 μl of the drug. Wells containing no drugs were used for control cell viability and wells containing no cells for blanking the spectrophotometer. A stock solution of 5 mg/ml MTT was prepared in saline and stored at −20 °C. After the cells were incubated at 37 °C for 3 days, an aliquot of 10 μl of MTT solution was added to each well and, after shaking for 1 min, the plate was incubated at 37 C for 5 h. Formazan crystals in the suspension culture were dissolved with 100 μl of 0.04 N HCl–isopropanol whereas those of the monolayer culture were dissolved with 150 μl of dimethyl sulfoxide (DMSO) following the removal of the supernatant. The optical density of the wells was measured with a microplate reader at 540 nm. The minimal inhibitory concentration (MIC) for a particular peptide was defined as the peptide concentration that results in a 100% reduction in the cell number compared to the untreated control. MIC values were determined directly from semilogarithmic dose–response curves. The experiment was carried out in triplicate, at least.

FACScan analysis. For analysis of the membrane integrity after peptide treatment, fungal cells (2×10^5 cells in YPD media) were first harvested at the log phase and mixed with the analog peptide (P6) at a concentration of 20 μM. The cells were incubated for a further 30 min at a physiological temperature of 28 °C with constant shaking (140 rpm). After incubation, the cells were harvested by centrifugation and washed three times with PBS. Permeabilization of the cell membrane was detected by incubation of the peptide treated cells in propidium iodide (PI, 50 μg/ml final concentration) at 4 °C for 30 min followed by removal of unbound dye through excessive washing with PBS. Flow cytometry was performed using a FACScan (Becton Dickinson, San Jose, CA).

Measurement of plasma membrane fluorescence anisotropy. Anisotropy of fluorescence from exponential *C. albicnas* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Eugene, Oregon, USA) was used to monitor changes in membrane dynamics. The cells (1×10^8 cells in YPD medium) containing the peptides, PMAP-23 and its analogs at various concentrations, were incubated at a physiological temperature of 28 °C on a rotary shaker at 150 rpm for 2 h. Control cells were incubated without peptides. The cells were fixed with paraformaldehyde (0.25% v/v) for 30 min. The fixed cells were then collected by centrifugation at 3000 rpm, washed several times with PBS buffer (pH 7.4), and the pellets were frozen in liquid nitrogen. For labeling of DPH, the pellets were resuspended in PBS buffer and incubated at 28 °C for 45 min in the presence of 0.6 mM DPH followed by several washings in PBS buffer. Steady-state fluorescence anisotropy was measured using a Perkin–Elmer LS50B spectrofluorometer (Perkin–Elmer Ltd, Beaconsfield, UK) at 350 nm excitation and 425 nm emission wavelengths [19].

Morphological changes observed by scanning electron microscopy after peptide treatment. Subcultured *C. albicans* cells were incubated at 28 °C for 4 h with 1.25 μM of PMAP-23 analog peptide (P6) and melittin. Negative controls were run in the absence of peptide solutions. The cells were fixed with an equal volume of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). After fixation for 3 h at 4 °C, the samples were centrifuged at 2000 rpm and washed twice with 0.05 M cacodylate buffer (pH 7.2). The samples were dehydrated with a graded ethanol series (50%, 70%, 90%, 95%, and 100% EtOH). After lyophilization and gold coating, the samples were examined on a HITACHI S-2400 (Tokyo, Japan) [20].

Effect of peptide on the dimorphic transition. C. albicans was maintained by periodic subculturing in liquid YPD medium. Cultures of yeast cells (blastoconidia) were maintained in liquid YPD medium at 37 °C. To induce hyphal formation, cultures were directly supplemented with 20% fetal bovine serum. The dimorphic transition in *C. albicans* was investigated from cultures containing various concentrations of peptide, incubated at 37 °C for 36 h. The dimorphic transition to hyphal forms was detected by phase contrast light microscopy (NIKON, ECLIPSE TE300, Japan) [21].

Results and discussion

Design and synthesis of the PMAP-23 analogs

The amphipathic feature of α-helical antimicrobial peptides plays an important role against target cells.

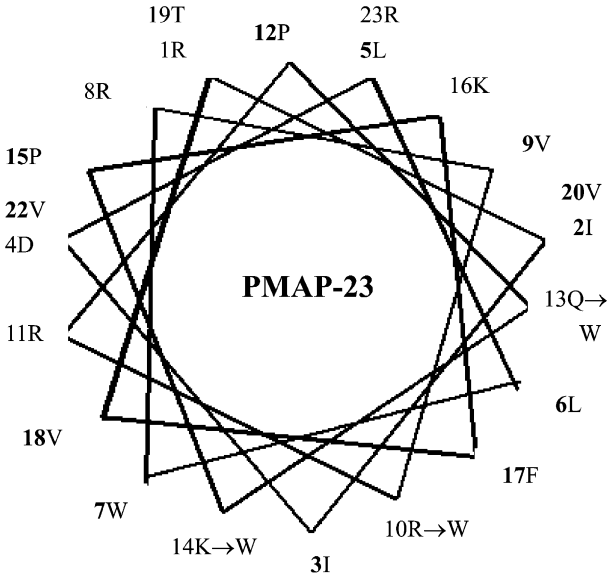

Fig. 1. The α-helical wheel diagram of PMAP-23. The bold indicates the hydrophobic amino acid residues and the arrows indicate the substituted tryptophan amino acid residues in PMAP-23.

Furthermore, a number of parameters, including net positive charge, α-helicity, and overall hydrophobicity, have been shown to modulate the antibiotic activity of α-helical amphipathic antimicrobial peptides [21,22]. This is the case with PMAP-23, which has a similar pattern, with both hydrophobic and hydrophilic regions, as shown in the α-helical wheel diagram (Fig. 1).

To elucidate the relationship between hydrophobic and hydrophilic regions of antibiotic peptides and to design novel peptides with more potent antibiotic activity than PMAP-23, a variety of analogs were designed and synthesized. These analogs were designed on the basis of the α-helical wheel diagram of PMAP-23 (Fig. 1). Substituting with tryptophan increased the hydrophobicity. The amino acid sequences used in this study are summarized in Table 1. The synthetic peptides were purified using reverse-phase (RP)-HPLC and the correct molecular weights of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 2).

RP-HPLC has often been employed to determine, experimentally, the quantitative hydrophobic–hydrophilic balance of amphipathic peptides [23,24]. Peptides separate on RP-HPLC due to their different hydrophobic interactions with the C-18 stationary phase. Such hydrophobic interactions can be considered comparable to the interaction of amphipathic antimicrobial peptides with the lipid bilayers of plasma membranes [25]. To investigate a possible correlation between the overall peptide hydrophobicity and fungicidal activities, tumor cells, and the hydrophobic characteristics of the peptides, were investigated by comparing their retention times on RP-HPLC. The mean hydrophobicities were calculated, using consensus values from hydrophobicity scales, for each amino acid residue [26] (Table 2). As expected, every analog eluted later than PMAP-23. In particular, Trp-substitutions at positions 10, 13, and 14 in PMAP-23

Table 1
Amino acid sequences of synthetic antimicrobial peptide, PMAP-23 and its analogs

Peptides	Amino acid sequences	Remarks
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR-NH_2	Native
P1	RIIDLLWRV**W**RPQKPKFVTVWVR-NH_2	($R^{10} \rightarrow W^{10}$)
P2	RIIDLLWRVRRP**W**KPKFVTVWVR-NH_2	($Q^{13} \rightarrow W^{13}$)
P3	RIIDLLWRVRRPQ**W**PKFVTVWVR-NH_2	($K^{14} \rightarrow W^{14}$)
P4	RIIDLLWRVRRP**WW**PKFVTVWVR-NH_2	($Q^{13}, K^{14} \rightarrow W^{13}, W^{14}$)
P5	RIIDLLWRV**W**RP**W**KPKFVTVWVR-NH_2	($R^{10}, Q^{13} \rightarrow W^{10}, W^{13}$)
P6	RIIDLLWRV**W**RP**WW**PKFVTVWVR-NH_2	($R^{10}, Q^{13}, K^{14} \rightarrow W^{10}, W^{13}, W^{14}$)

Table 2
Molecular weights of the peptides determined by MALDI mass spectrometry and their mean hydrophobicities

Peptides	Observed value	Calculated value	Retention time (min)	Mean hydrophobicity (H)
PMAP-23	2962.5	2961.9	21.858	−6.90
P1	2991.9	2991.9	23.722	−4.73
P2	3020.0	3020.9	22.892	−5.84
P3	3019.9	3019.7	24.248	−5.43
P4	3078.0	3078.2	25.127	−4.37
P5	3050.0	3049.4	25.645	−3.67
P6	3108.0	3107.6	27.890	−2.20

The mean values were calculated using consensus values of hydrophobicity scale for each amino acid residue.

analog peptide (P6) were revealed to have the most increased hydrophobicities compared to the other analogs.

Fungicidal and antitumor activities of PMAP-23 analogs

In our previous study, PMAP-23 was shown to inhibit the growth of fungi and while there have been no extensive studies on the effects of PMAP-23 on lipid vesicles, its effect on fungal cell membranes has been completely ignored [14]. In this study, the fungicidal activities of the synthetic peptides, against pathogenic fungal strains, were determined as the MIC, using the microdilution method [27] (Table 3). With a few of the amino acid residues, Trp-substitutions at positions 10, 13, and 14 in PMAP-23 (P1, 2, 3, 4, 5) did not reveal any significantly improved fungicidal activity, compared with the PMAP-23. In particular, all the substitutions of Arg, Gln, and Lys for hydrophobic amino acids, and Trp at positions 10, 13, and 14 of PMAP-23 analog peptide (P6), caused a dramatic increase in the fungicidal activity. The result suggests that the increased hydrophobicity of synthetic peptides has an important role in the fungicidal activity.

Previously, Magainin 2, Cecropin A, and Cecropin B were found to be toxic to tumor cells at concentrations lower than those required to lyse normal fibroblasts [28–30]. Therefore, the antitumor activities of the peptides designed in this study, against Jurkat and SNU601 tumor cells, were examined using the MTT assay. As shown in Table 4, PMAP-23 had some antitumor activity against the two tumor cell lines. Meanwhile, P6 displayed about fourfold greater antitumor activity than the parent PMAP-23. This PMAP-23 analog P6 peptide was henceforth referred to as PMAP-23/P6.

Table 3
Fungicidal activities of PMAP-23 and its analog peptides

Peptides	MIC (μM)		
	C. albicans	*T. beigelii*	*S. cerevisiae*
PMAP-23	20	20	10–20
P1	20	10	10
P2	10–20	10	10
P3	10–20	10–20	10–20
P4	10	20	10–20
P5	10	20	20
P6	2.5–5	5–10	2.5–5

Table 4
Antitumor activities of PMAP-23 and its analogue peptides

Peptides	IC50 (μM)	
	Jurkat	SNU 601
PMAP-23	35	47
P1	19	28
P2	20	30
P3	29	39
P4	23	37
P5	16	21
P6	11	10

Effects of salts and sodium azide on fungicidal activity of PMAP-23/P6

Recently, it has been proposed that the ion channel raises the concentration of salt on the epithelial surface and this high salt concentration inhibits the activity of antimicrobial peptides. To evaluate whether salts influence the fungicidal activity of synthetic peptides, the effects of calcium chloride and magnesium citrate, on the fungicidal effect of PMAP-23/P6, were investigated using FACScan (Becton Dickinson, San Jose, CA). The fungicidal activity of PMAP-23/P6, against *C. albicans,* was remarkably reduced (Fig. 2).

Cells were incubated with the PMAP-23/P6, at 28 °C for 4 h in the absence, or presence, of NaN_3 as a respiratory inhibitor. The *C. albicans* cells showing PI uptake were analyzed by a flow cytometer. As shown in Fig. 3, the PMAP-23/P6 was highly dependent on the presence of the respiration inhibitor, NaN_3. It was suggested that the fungicidal activity of the PMAP-23/P6 was mediated by a cellular function requiring cellular energy consumption.

Effect of PMAP-23/P6 on plasma membrane and cell morphology

The mechanism for the action of PMAP-23/P6 was further investigated using DPH as a membrane probe. If the fungicidal activities exerted by the PMAP-23/P6, on *C. albicans*, are at the level of the plasma membrane, DPH, that interacts with an acyl group of plasma membrane lipid bilayers, could not be inserted into the membrane. As shown in Fig. 4, increasing fungicidal activity of the peptide significantly decreased plasma membrane DPH fluorescence anisotropy. This suggests that exponentially grown cultures, exposed to moderate peptide concentrations, exhibit a structurally perturbed plasma membrane.

We also examined morphological changes induced by the PMAP-23/P6 by scanning electron microscopy. Untreated *C. albicans* had a normal, smooth surface (Fig. 5A). In contrast, cells treated for 4 h with PMAP-23/P6 and melittin showed surface roughening and disrupting (Figs. 5B and C, respectively). The SEM observations provide morphological evidence of the potent permeabilizing activity of the PMAP-23/P6. The

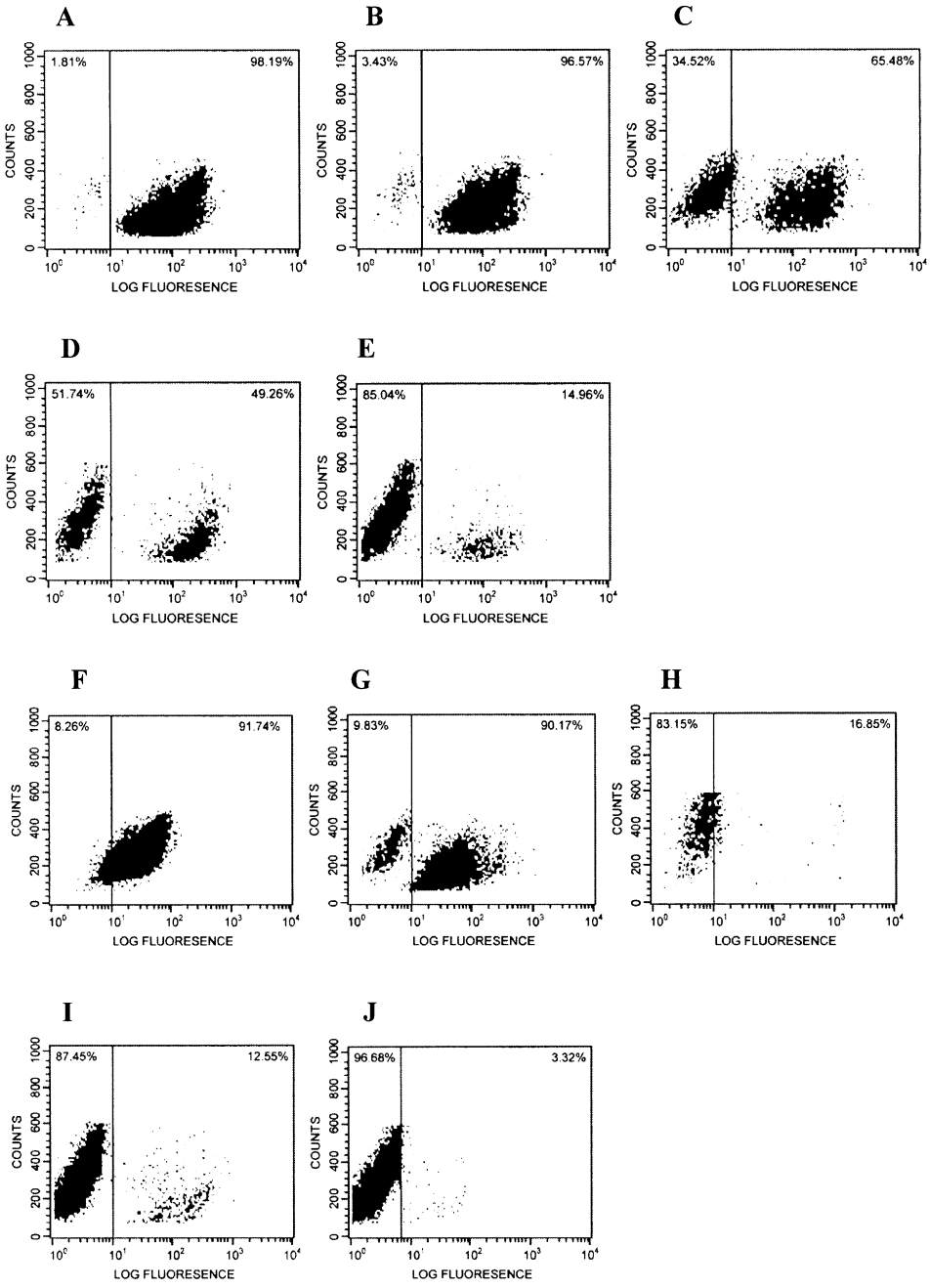

Fig. 2. Salt-dependency of PMAP-23/P6. Exponential phase *C. albicans* cells were treated with 25 μM of the PMAP-23/P6 or 25 μM of melittin. The cell population showing fluorescence was analyzed by the FACScalibur flow cytometer. The increments of the log fluorescence signal represent PI uptake by peptides. (A)–(E) present the PMAP-23/P6-treated cells with: (A) no salts, (B) 20 mM NaCl, (C) 100 mM NaCl, (D) 20 mM $MgCl_2$, and (E) 100 mM $MgCl_2$. (F)–(J) represent the melittin-treated cells with: (F) no salts, (G) 20 mM NaCl, (H) 100 mM NaCl, (I) 20 mM $MgCl_2$, and (J) 100 mM $MgCl_2$.

cell membrane alterations are similar to those induced by melittin used as a positive control.

The ability of some fungi to undergo a morphological transition between unicellular forms and hyphae structures may be considered a simple model of cellular development. *C. albicans* is prototypic dimorphic yeast. This diploid pathogen is of increasing importance in human medicine. In *C. albicans*, dimorphism plays a crucial role in pathogenesis, with mycelial shapes being predominantly found during host tissue invasion [31]. To induce filamentation, cultures were directly supplemented with serum. To investigate the effect of the PMAP-23/P6 on the dimorphic transition of *C. albicans*, this transition was

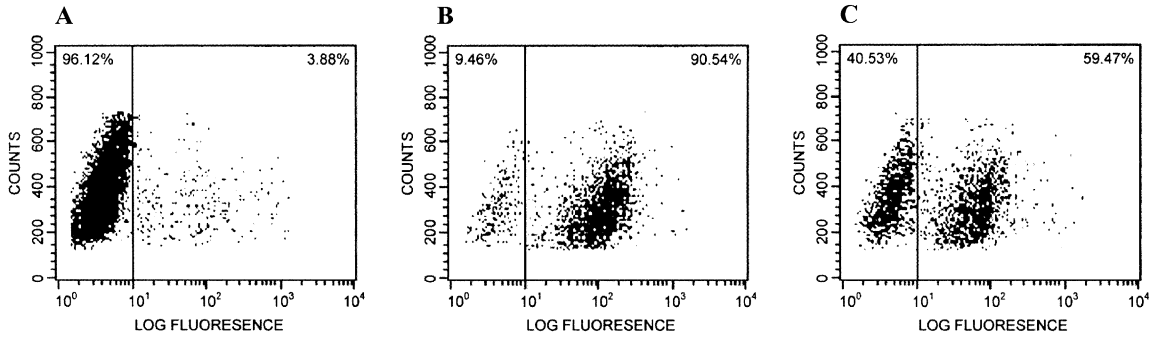

Fig. 3. The effect of sodium azide (NaN_3) on the PMAP-23/P6. Exponential phase *C. albicans* cells were treated with 25 μM of the PMAP-23/P6. The cell population showing fluorescence was analyzed by the FACScalibur flow cytometer. The increments of the log fluorescence signal represent PI uptake by peptides: (A) control, (B) PMAP-23/P6-treated cells, (C) PMAP-23/P6 and NaN_3-treated cells.

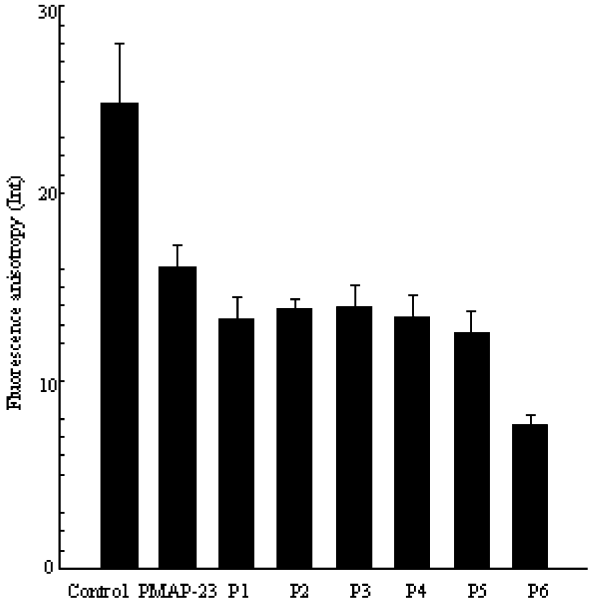

Fig. 4. DPH fluorescence anisotropy after the addition of peptides, PMAP-23 and its analogs. For labeling, 10^{-7} M DPH (added as 10^{-4} M solution in tetrahydrofuran) was added to resuspended cells and then the plasma membrane was purified by sonication.

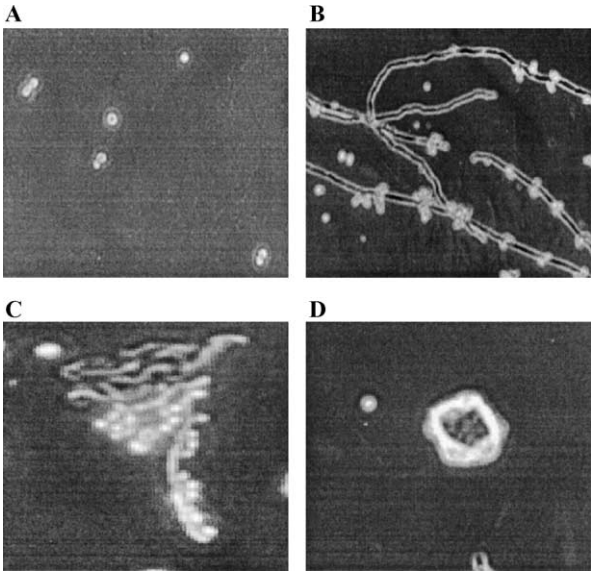

Fig. 6. The effect of the PMAP-23/P6 on the dimorphic transition in *C. albicans*. Each culture was incubated with various concentrations (10, 20 μM) of the PMAP-23/P6 for 48 h in YPD media with 20% FBS: (A) yeast control with no 20% FBS and peptide, (B) with no treated peptide, (C) with 10 μM PMAP-23/P6, and (D) 20 M PMAP-23/P6.

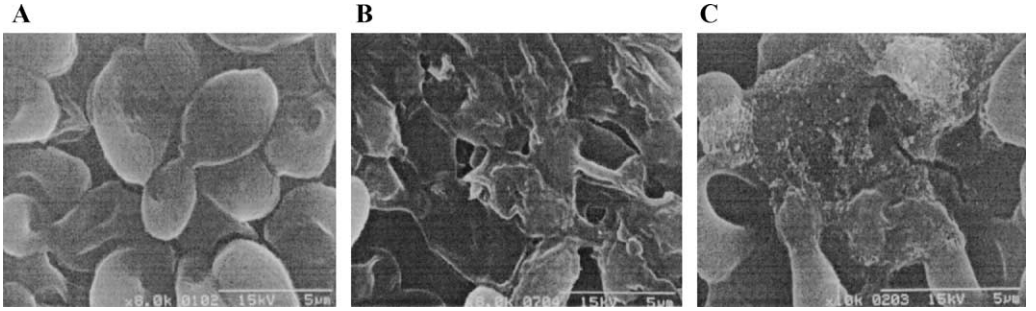

Fig. 5. Scanning electron microscopy of: (A) untreated *C. albicans* and after treatment for 4 h at 28 °C, (B) with 12.5 μM melittin, and (C) 12.5 μM PMAP-23/P6.

examined in cultures containing various concentrations of peptide, incubated with fetal bovine serum at 37 °C for 36 h. Fig. 6 shows that the peptide destroyed the hyphal forms at 10 and 20 μM peptide concentrations, indicating that the PMAP-23/P6 could cause the disruption of the serum-induced filamentous form of *C. albicans*.

Conclusion

To obtain antibiotic peptides useful for pharmaceutical applications, a strong antibiotic activity is required against bacterial, fungal, or cancer cells. In the present study, we synthesized six analogs, designed to increase the hydrophobic helical face, by substituting hydrophobic amino acids with Trp from PMAP-23, derived from the porcine myeloid. In particular, the PMAP-23/P6, which substituted Arg, Gln, and Asp with Trp at positions 10, 13, and 14 of PMAP-23 caused a dramatic increase in the antibiotic activity. This PMAP-23/P6, which exhibits potent fungicidal and antitumor activities, may have potential as a specific pharmacological agent, as a model for the study of the hydrophobicity–antibiotic relationship of peptides, as well as in the development of a novel therapeutic agent.

Acknowledgments

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