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A Leu–Lys-rich antimicrobial peptide: activity and mechanism

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Abstract

To develop novel antibiotic peptides useful as therapeutic drugs, the analogues were designed to increase not only net positive charge by Lys substitution but also hydrophobic helix region by Leu substitution from cecropin A (1–8)–magainin 2 (1–12) hybrid peptide (CA–MA). In particular, CA–MA analogue P5 (P5), designed by flexible region (GIG → P) substitution, Lys (positions 4, 8, 14, 15) and Leu (positions 5, 6, 12, 13, 16, 17, 20) substitutions, showed an enhanced antimicrobial and antitumor activity without hemolysis. Confocal microscopy showed that P5 was located in the plasma membrane. The antibacterial effects of analogues were further confirmed by using 1,6-diphenyl-1,3,5-hexatriene as a plasma membrane probe. Flow cytometric analysis revealed that P5 acted in an energy-independent manner. This interaction is also independent of the ionic environment. Furthermore, P5 causes significant morphological alterations of the bacterial surfaces as shown by scanning electron microscopy and showed strong membrane disrupting activity when examined using liposomes (phosphatidyl choline/cholesterol; 10:1, w/w). Its potent antibiotic activity suggests that P5 is an excellent candidate as a lead compound for the development of novel antiinfective agents.

Keywords: Cecropin A (1–8)–magainin 2 (1–12) hybrid peptide (CA–MA); CA–MA analogue P5 (P5); Lys and Leu substitution

1. Introduction

During the past decade, antibacterial peptides have been found in a wide range of species [1–10]. These antibacterial peptides are known to be important components of innate immunity and the host defense system of insects, amphibians and mammals [11,12]. Antibacterial peptides can be classified into cytotoxic peptides such as melittin (ME) that can lyse mammalian cells as well as bacterial cells [13] and peptides such as cecropin A (CA) [7,14,15] and magainin 2 (MA) [6] that are active only on bacterial cells. CA, a cationic 37-amino acid antimicrobial peptide, was isolated from *Hyalaphora cecropia* pupae [14] and MA, a cationic 23-amino acid antimicrobial peptide, was discovered from the skin of the African clawed frog, *Xenopus laevis* [6]. Both CA and MA exhibit strong antibacterial activity but no cytotoxicity against normal mammalian cells.

Although the precise mechanism of the antibacterial action of these amphipathic peptides is not yet entirely understood, recent studies suggest that the action is involved in the formation of ion channels with consequent disruption of bacterial phospholipid bilayers and eventual cell death [16–19].

Numerous studies using synthetic peptides have been focused on designing analogue peptides having more potent antimicrobial activity than that of natural peptides without damaging against mammalian cells [20,21]. Several attempts have been made to improve antimicrobial activity against bacterial cells while eliminating the cytotoxicity against mammalian cells such as red blood cells by flexible region [22], chain length [23], chaining the net charge [24,25], hydrophobicity [8] and/or α-helicity [26].

In this study, novel analogue peptides by chain-length deletion and increasing net positive charge and hydrophobicity were designed and synthesized from the sequence of

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CA–MA. To investigate the correlation between antibiotic activity and the Leu–Lys-rich model peptide, the antimicrobial and anticancer activities were measured against bacterial, fungal and cancer cells. Additionally, we will discuss the importance of Leu–Lys-rich peptide, the antibacterial effect of analogues using the membrane probe and its effectiveness on the damage in the cell membrane.

2. Materials and methods

2.1. Peptide synthesis

The peptides were synthesized by the solid-phase method using 9-fluorenyl-methoxycarbonyl (Fmoc) chemistry [27]. 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-L-amino acids was performed with *N*-hydroxybenzotriazole (HoBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp and Thr), trityl (Gln), *tert*-butyloxycarbonyl (Lys and Trp), pmc (Arg). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol and triisopropylsilane (82.5:5:5:5:2.5:2 v/v) for 3 h at room temperature. The crude peptide was then repeatedly washed with diethylether, and dried in a vacuum. The crude peptides were purified by a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19 × 30 cm). The purified peptides were hydrolyzed with 6 N HCl at 110 °C for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 N HCl and subjected to an amino acid analyzer (Hitachi Model, 8500 A, Hitachi, Tokyo, Japan). Peptide concentration was determined by amino acid analysis. The molecular masses of the peptides were confirmed with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS).

2.2. Antibacterial activity

Bacillus subtilis (KCTC 1918), *Streptococcus epidermidis* (KCTC 3096), *Pseudomonas aeruginosa* (KCTC 1637) and *Salmonella typhimurium* (KCTC 1926) were supplied by the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejon, Korea. The bacteria were grown to the mid-phase in a medium (g/l) (10 bactotryptone/5 yeast extract/10 NaCl, pH 7.0). The peptides were filtrated through a 0.22-μm filter and stepwise diluted in a medium of 1% bactopeptone. The tested organism [final bacterial suspension: 2×10^6 colony-forming units (CFU)/ml] suspended in growth medium (100 μl) was mixed with 100 μl of the 2-fold-diluted serial solution of each peptide in a microtiter plate well with three replicates for each test sample. The plates were incubated for 18 h at 37 °C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide that gave no visible growth on the plate [26].

2.3. Antifungal activity assay

The fungal strains, *Candida albicans* (TIMM 1768) and *Trichosporon beigelii* (KCTC 7707) were seeded on 96-well plates (NUNC) at a density of 2×10^4 cells per well in a volume of 100 μl of YPD media (dextrose 2%, peptone 1%, yeast extract 0.5%, pH 5.5), respectively. *T. beigelii* (KCTC 7707) was obtained from the KCTC, KRIBB. *C. albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan. To these fungal cells were added each 100 μl of serially diluted peptides, and the cell suspension was incubated for 24 h at 28 °C. After incubation, 5 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 at 37 °C for a further 4 h. The optical density of each well was measured at 580 nm by a microtiter ELISA reader (Molecular Devices Emax, Sunnyvale, CA) [1].

2.4. Antitumor activity assay

Growth inhibitory activity of the peptides against cancer and human T-cell lymphoma cells was determined as inhibition concentration (IC) using a tetrazolium (MTT) colorimetric assay. Human stomach cancer (SNU601: KCLB cat. no. 00601), unknown, probably lung carcinoma (Calu-6: KCLB cat. no. 30056) and human acute T-cell leukemia (Jurkat: ATCC cat no. TIB-152) were used for the growth inhibitory activity assay of the peptides against cancer cells. SNU601 and Calu-6 cells were purchased from the Cell Bank of Seoul National University, Korea. Also, Jurkat cells were obtained from the Genetic Resources Center of KRIBB (Taejon, Korea). The cells were grown in an RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The cells were plated on 96-well plates at a density of 2.0×10^4 cells/well in 150 μl of the same medium. After incubating the plates overnight at 37 °C in 5% CO_2 atmosphere, 20 μl of serially diluted peptides (from 100 to 0.78 μM) were added and then incubated for 3 days. Ten microliters of MTT solution (5 mg/ml MTT in PBS) was added to each well and the plates were incubated at 37 °C for 4 h. Forty microliters of 20% SDS solution containing 0.02 M HCl was added to dissolve the dark-blue crystals, which formed (MTT-formazan product) in each well, and then incubated overnight. Absorbance was measured at 570 nm on an ELISA plate reader (Molecular Devices Emax).

Table 1
Amino acid sequence and molecular masses determined by MALDI-MS of CA–MA hybrid peptide and its analogues

Peptides	Amino acid sequence	Remarks	Calculated values	Observed values
CA–MA	KWKLFKKIGIGKFLHSAKKF-NH_2	Parent peptide	2402.48	2403.01
P1	KWKLFKKIGIGKFL-NH_2	CA–MA: C-terminal deletion (HSAKKF)	1704.10	1706.00
P2	**KKK**KWKLFKKIGIGKFL-NH_2	P1: N-terminal addition (KKK)	2088.30	2090.03
P3	KWKLFKKIGIGKFL**KKK**-NH_2	P1: C-terminal addition (KKK)	2088.38	2090.05
P4	KWK**KK**KK**KP**KFL-NH_2	CA–MA: C-terminal deletion (HSAKKF) and substitution of GIG → Pro $L^4 \rightarrow K^4$, $F^5 \rightarrow K^5$, $I^8 \rightarrow K^8$	1585.07	1586.00
P5	KWK**KLLK**K**PLLKKLL**KK**L**-NH_2	CA–MA: substitution of GIG → Pro $L^4 \rightarrow K^4$, $F^5 \rightarrow L^5$, $K^6 \rightarrow L^6$, $I^8 \rightarrow K^8$, $K^{12} \rightarrow L^{12}$, $F^{13} \rightarrow L^{13}$, $L^{14} \rightarrow K^{14}$, $H^{15} \rightarrow K^{15}$, $S^{16} \rightarrow L^{16}$, $A^{17} \rightarrow L^{17}$, $F^{20} \rightarrow L^{20}$	2244.60	2246.03

The several analogues were designed to increase net positive charge and hydrophobicity by Lys and Leu substitutions, Lys addition, flexible region (Gly-Ile-Gly → Pro) substitution or C-terminal region (HSAKKF) deletion maintaining the tryptophan residue at position 2.

2.5. Hemolytic activity

The hemolytic activity of the peptides was evaluated by determining the released hemoglobin of 8% suspensions of fresh human erythrocytes at 414 nm [23]. Human red blood cells were centrifuged and washed three times with PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.0). One hundred microliters of human red blood cells suspended 8% (v/v) in PBS was plated into 96-well plates, and then 100 μl of the peptide solution (from 100 to 0.195 μM) was added to each well. The plates were incubated for 1 h at 37 °C, and centrifuged at $150 \times g$ for 5 min. One hundred-microliter aliquots of the supernatant were transferred to 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax). Zero percent and 100% hemolysis was determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation:

$$\begin{aligned}\text{percentage hemolysis} &= [(\text{Abs}_{414\ \text{nm}} \text{ in the peptide solution} \\ &\quad - \text{Abs}_{414\ \text{nm}} \text{ in PBS}) \\ &\quad /(\text{Abs}_{414\ \text{nm}} \text{ in 0.1\% Triton X-100} \\ &\quad - \text{Abs}_{414\ \text{nm}} \text{ in PBS})]100.\end{aligned}$$

2.6. Kinetics of bacterial killing

The kinetics of bacterial killing of the peptides was evaluated using *B. subtilis* and *P. aeruginosa*. Log-phase bacteria (6×10^5 CFU/ml) were incubated with 0.78 μM peptide in 1% bactopeptone media. Aliquots were removed

Table 2
Antimicrobial activity of CA–MA hybrid peptide and its analogues

Peptides	MIC: μM					
	Gram-positive bacteria		Gram-negative bacteria		Fungal cells	
	B. subtilis	*S. epidermidis*	*P. aeruginosa*	*S. typhimurium*	*C. albicans*	*T. beigelii*
CA–MA	3.12	1.56–3.12	1.56	0.19	12.5	6.25
P1	6.25	6.25	3.12	1.56	12.5	6.25–12.5
P2	3.12	3.12	1.56	0.19	12.5	6.25
P3	3.12	3.12	1.56–3.12	0.19	12.5	6.25
P4	6.25	12.5	3.12	1.56–3.12	>25	>25
P5	0.78	1.56	0.78–1.56	0.097	6.25	3.25

The bacteria were grown to the mid-logarithmic phase in a medium (g/l) (10 bactotryptone/5 yeast extract/10 NaCl, pH 7.0). Microbial growth was determined by the increase in optical density at 620 nm after 10 h incubation at 37 °C.
The fungal strains were grown at 30 °C in YPD media (dextrose 2%, peptone 1%, yeast extract 0.5%, pH 5.5). The fungal cells were seeded on the well of a 96-microtiter plate of YPD media at a density of 2×10^3 cells (100 μl/well). The turbidity of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices Emax).

at fixed time intervals, appropriately diluted, plated on the agar plate, and then the CFU were counted after 16 h incubation at 37 °C.

2.7. Confocal laser scanning microscopy

Intracellular localization of the fluorescein isothiocyanate (FITC)-labeled P5 in *C. albicans* was analyzed by confocal laser scanning microscopy (CLSM). *C. albicans* cells were inoculated into 3 ml of yeast medium and incubated at 28 °C for 12 h. The cells were then diluted 1:50 in Saubouraud dextrose broth medium (SB medium; 1% bacto peptone, 4% glucose) and incubated at 28 °C for 3 h to enrich the population of exponentially growing cells. The number of cells was adjusted to 10^6 cells per milliliter by diluting in the SB medium. In some cases, the stationary-phase cells were used instead of the exponential-phase cells. FITC-P5 was added to 100 μl of the cell suspension at 6.25 μM, and the cells were incubated at 28 °C for 15 min. The cells were pelleted by centrifugation at 10,000 rpm for 5 min and washed three times with ice-cold PBS buffer. Intracellular localization of FITC-P5 was examined by the Leica TCS 4D connected to an Olympus IX 70 upright microscope (Olympus).

2.8. Measurement of plasma membrane fluorescence anisotropy

Fluorescence from *B. subtilis* and *P. aeruginosa* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH, Molecular Probes, Inc., Eugene, OR) was used to monitor changes in membrane dynamics. The labeling and fluorescence measurements were performed exactly as described [28]. Briefly, samples of the bacterial culture (3 ml) were fixed by formaldehyde (0.25% final concentration), collected in Eppendorf tubes and washed with PBS, and the cells frozen in liquid nitrogen. For labeling, cells were thawed, bacteria resuspended in PBS ($OD_{450} = 0.25$) and incubated for 45 min at 37 °C in the presence of 10^{-7} M DPH (added as 10^{-4} M solution in tetrahydrofuran). Steady-state fluorescence anisotropy was measured at 37 °C by Hitachi F-3010 spectrofluorometer (Hitachi). The excitation wavelength was set to 330 nm. Fluorescence was detected through a cutoff emission filter and the emission monochromator was set to 450 nm. The steady-state anisotropy was calculated according to Ref. [28].

2.9. Energy- and salt-dependent test

For analysis of the membrane integrity after peptide treatment, *C. albicans* cells (2×10^5 cells in YPD media) were first harvested at log phase. Peptides were added to a final concentration of 20 μM and the 0.05% NaN_3 as a respiration inhibitor and sodium chloride (final concentrations; 0 and 150 mM) were added for energy- and salt-dependent test. The cells were incubated for a further 30 min at physiological temperature of 28 °C under 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. The fluorescence of PI was monitored in the FL2-H channel. As

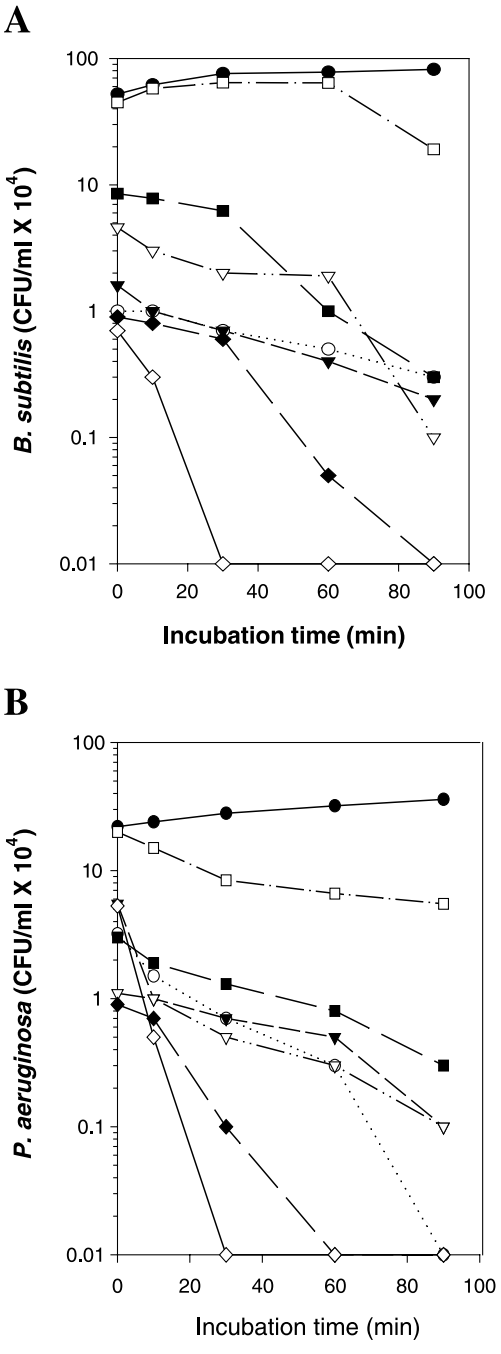

Fig. 1. Time killing plots for *B. subtilis* (A) and *P. aeruginosa* (B) by CA–MA and its analogues. The kinetics of bacterial killing of the peptides was evaluated using *B. subtilis* and *P. aeruginosa*. Log-phase bacteria (2×10^6 CFU/ml) were incubated with 2.5 μM peptide in 1% bactopepton. No treated peptide (●), CA–MA (○), P1 (▼), P2 (▽), P3 (■), P4 (□), P5 (♦), melittin (◇).

a control experiment, the cell suspension was treated with melittin. Flow cytometric analysis was performed by the FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.10. Scanning electron microscopy (SEM)

Midlog phase *B. subtillis* or *P. aeruginosa* were resuspended at 10^8 CFU/ml in Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37 °C with CA–MA and P5. Controls were run in the absence of peptide solvent. After 30 min, the cells were fixed with an equal volume of 5% glutaraldehyde in 0.2 M Na-cacodylate buffer, pH 7.4. After fixation for 2 h at 4 °C, the samples were filtered on Isopore filters (0.2 μm pore size, Millipore, Bedford, MA) and extensively washed with 0.1 M Na-cacodylate buffer, pH 7.4. The filters were then treated with 1% osmium tetroxide, washed with 5% sucrose in cacodylate buffer and subsequently dehydrated with a graded ethanol series. After lyophilization and gold coating, the samples were examined on a Hitachi S-2400 instrument (Hitachi).

2.11. Preparation of small unilamellar vesicles (SUVs)

SUVs were prepared by drying phosphatidyl choline (PC)/cholesterol (10:1, w/w) under nitrogen, suspending 100 mM of the film in 50 mM of phosphate buffer at pH 7.5, mixing by vortex and sonicating using the tip of an ultrasonic probe. A drop was deposited on a carbon-coated grid and negatively stained with 2% uranyl acetate. SUVs were mixed with the peptide at a concentration of 125 nM.

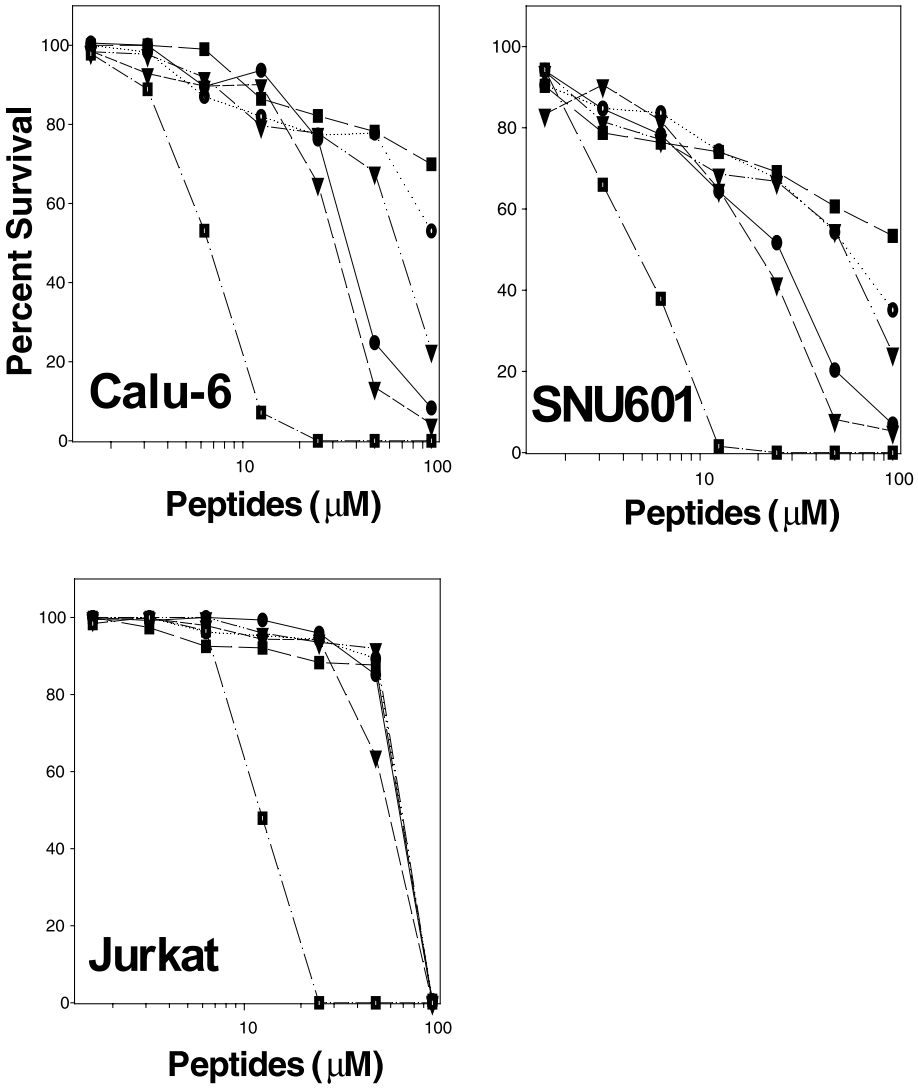

Fig. 2. Concentration–response curves of the peptides in the growth inhibition against three different transformed tumor cells. The in vitro cytotoxicity of peptides was determined using the MTT assay. Cells were seeded at 2×10^5 cells/ml in α-MEM containing 10% FBS. Symbols: ●, CA–MA; ○, P1; ▼, P2; ▽, P3; ■, P4; □, P5.

Specimens were examined in a TECNAI 12 (Philips) at an accelerating voltage of 120 kV.

3. Results and discussion

3.1. Design and synthesis of the peptides

Generally, the amphipathic feature of α-helical antimicrobial peptides plays an important role against target cells. In addition, a number of parameters, including net positive charge, α-helicity, and overall hydrophobicity have also been shown to modulate the antibiotic activity of the α-helical amphipathic antimicrobial peptides [23].

Previously, we reported the hybrid peptide designed from N-terminal amphipathic region of CA and hydrophobic region of MA [23]. This hybrid peptide, CA (1–8)–MA (1–12) showed increased antimicrobial activity than CA or MA. In this study, to elucidate the relationship between chain length, electrostatic property, hydrophobicity of antibiotic peptides, and to design peptides with increased antibiotic activity than CA–MA without cytotoxicity, certain analogues of CA–MA were designed and synthesized. The amino acid sequences used in this study were summarized and the correct molecular weights of the synthetic peptides were confirmed by MALDI-MS (Table 1). The several analogues were designed to increase net positive charge and hydrophobicity by Lys and Leu substitutions, Lys addition, flexible region (Gly-Ile-Gly → Pro) substitution or C-terminal region (HSAKKF) deletion maintaining the tryptophan residue at position 2. In particular, CA–MA analogue P5 (P5), designed by flexible region (GIG → P) substitution, Lys (positions 4, 8, 14, 15) and Leu (positions 5, 6, 12, 13, 16, 17, 20) substitutions. Because it was shown in our previous study that the flexible region (Gly-Ile-Gly → Pro) substitution did not affect the α-helical structure and the antibacterial activity [22], but the tryptophan residue in position 2 of the CA–MA was critical in antibacterial activity [29].

3.2. Antimicrobial and mammalian cell toxicity

Antibacterial activities of the synthetic peptides against Gram-positive and Gram-negative bacterial strains were determined as the MIC by the microdilution method [26] (Table 2). The flexible region (GIG → P) substitution, Lys (positions 4, 8, 14, 15) and Leu (positions 5, 6, 12, 13, 16, 17, 20) substitutions (P5) showed strong antibacterial activity with a MIC value of 0.097–1.56 μM. Both C-terminal (HSAKKF) deletion and net positive charge additions [N-terminal (KKK, analogue P2) or C-terminal (KKK, analogue P3)] induced a similar or 2-fold decrease in the antibacterial activity against all the bacterial cells tested. The C-terminal (HSAKKF) deletion and substitution ($L^4 \rightarrow K^4$, $F^5 \rightarrow K^5$ and $I^8 \rightarrow K^8$) analogues P1 and P4 were 2 or 16 times less active than CA–MA. Also, the times required for the synthetic peptides to kill mid-logarithmic phase of *P. aeruginosa* and *B. subtilis* were compared. As shown in Fig. 1, P5 and melittin, used as a comparison, displayed faster bactericidal rates against both *P. aeruginosa* and *B. subtilis* than other analogues. This result indicates that the positive charge and hydrophobicity of synthetic peptide are important in determining the bactericidal rate and activity. The antifungal activity of the peptides against the pathogenic fungi was measured as MIC by MTT assay [1]. The results indicated that P5 displayed approximately 2-fold greater antifungal activity than CA–MA against fungal cells (Table 2). This result is similar to antibacterial activity. The results indicate that the order of antimicrobial activity of the analogue peptides used in this study was P5>CA–MA>P2 = P3>P1>P4.

On the other hand, magainin 2, cecropin A or cecropin B were known to be toxic against cancer cells at concentrations lower than required to lyse normal fibroblast [1] or human erythrocytes [1,6]. Therefore, the anticancer activity of the peptides designed in this study against Calu-6, SNU601 and Jurkat cancer cells was examined by MTT assay. As shown in Fig. 2, the analogue peptides showed activity against the three cancer cell lines. In particular, P5 showed a stronger anticancer activity than CA–MA. To assess the cytotoxicity of the peptides against mammalian cells, the percentage hemolysis was measured against human erythrocytes at various peptide concentrations (from 0.195 to 100 μM), and the synthetic peptides used in this study showed no hemolytic activity, while melittin, a honeybee venom toxin, has also been reported to possess potent antimicrobial activity over a broader spectrum [13], exhibiting a strong hemolytic activity (Table 3). In summary, the P5, which showed most remarkable antibiotic activity with no hemolytic activity among all the analogues, selectively kills bacterial, fungal and cancer cells while maintaining no hemolytic activity against human erythrocyte cells. These results indicate that the proper chain length,

Table 3
Hemolytic activity of CA–MA hybrid peptide and its analogues

Peptides	Percentage hemolysis (μM)									
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.195
CA–MA	0	0	0	0	0	0	0	0	0	0
P1	0	0	0	0	0	0	0	0	0	0
P2	0	0	0	0	0	0	0	0	0	0
P3	0	0	0	0	0	0	0	0	0	0
P4	0	0	0	0	0	0	0	0	0	0
P5	0	0	0	0	0	0	0	0	0	0
Melittin	100	100	100	100	100	95	93	31	0	0

The hemolytic activity of the peptides was evaluated by determining the hemoglobin release of 8% suspensions of fresh human erythrocytes at 414 nm. The hemolysis percentage was calculated using the following equation: percentage hemolysis=[($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)]100.

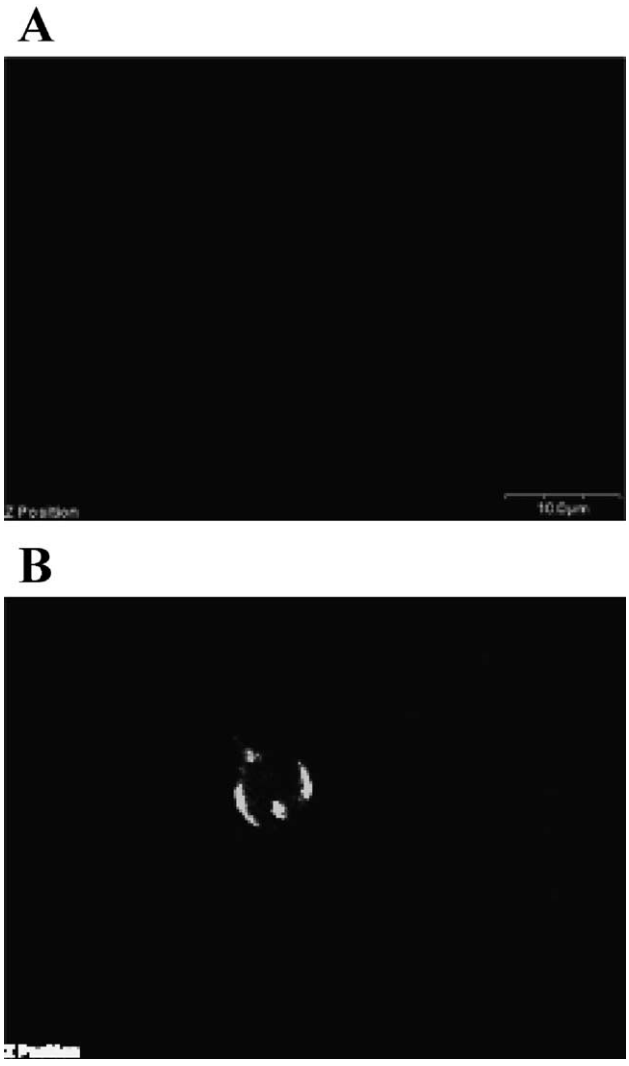

Fig. 3. Confocal fluorescence microscopy of *C. albicans* cells treated with FITC-P5. Cells treated with FITC-labeled P5 were incubated for 15 min at 28 °C. Visualization and localization of the labeled peptide was performed using Leica TCS 4D connected to an Olympus IX 70 upright microscope (Olympus, Japan). (A) No treated peptide; (B) FITC-labeled P5.

positive charge and hydrophobicity play an important role in antibiotic activity without hemolytic activity.

3.3. Peptide interaction upon the membrane

To examine the target sites of P5 in *C. albicans*, the peptide was labeled with FITC and visualized under the confocal microscopy. FITC did not give any affect on the antimicrobial activity of P5. The FITC-labeled P5 penetrated the cell membrane and accumulated in the plasma membrane of the cell immediately after addition to the cells (Fig. 3). This result suggested that the major target site of P5 is the plasma membrane of the microorganisms.

The antibacterial activity of CA–MA and analogues were further investigated using DPH as a membrane probe. If the bactericidal activities exerted by the CA–MA and analogues, on *P. aeruginosa* and *B. subtilis*, are at the level of the plasma membrane, DPH, which interacts with an acyl group of plasma membrane lipid bilayers, could not be inserted into the membrane. As shown in Fig 4, increasing bactericidal activity of the peptide significantly decreased plasma membrane DPH

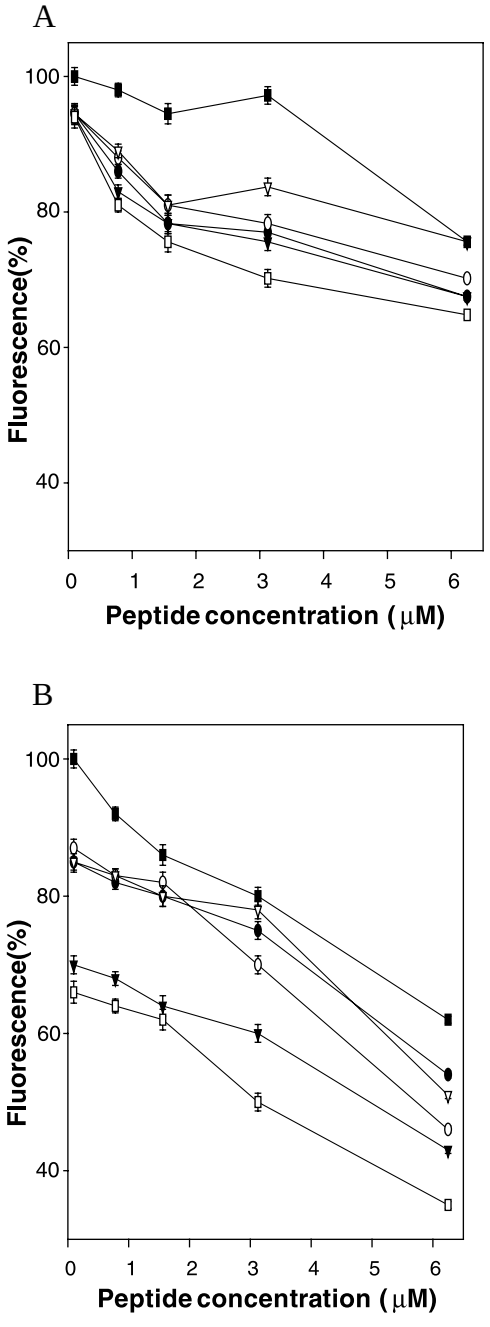

Fig. 4. Measurement of fluorescence by DPH labeling in in vivo membrane. Steady-state fluorescence anisotropy was measured at 37 °C by HITACHI F-3010 spectrofluorometer. The excitation wavelength was set to 330 nm. Fluorescence was detected through a cutoff emission filter and the emission monochromator was set to 450 nm. (A) *B. subtilis*; (B) *P. aeruginosa*. CA–MA (●), P1 (○), P2 (▼), P3 (▽), P4 (■), P5 (□).

fluorescence anisotropy. This suggests that when exposed to moderate peptide concentrations, plasma membrane was structurally perturbed in exponentially grown cultures. These results suggest that the antimicrobial activities of these synthetic peptides, in terms of perturbing the lipid membrane, correlate with their DPH anisotropic activities, which also correlates with the antimicrobial activities of these peptides.

3.4. Effects of sodium azide and salt on antimicrobial activity

C. albicans cells were incubated with the CA–MA and P5 in the absence or presence of NaN_3 as a respiratory inhibitor for energy-dependency test by FACScan analysis. As shown in Fig. 5, the interaction of CA–MA with *C. albicans* cells, as observed with PI uptake

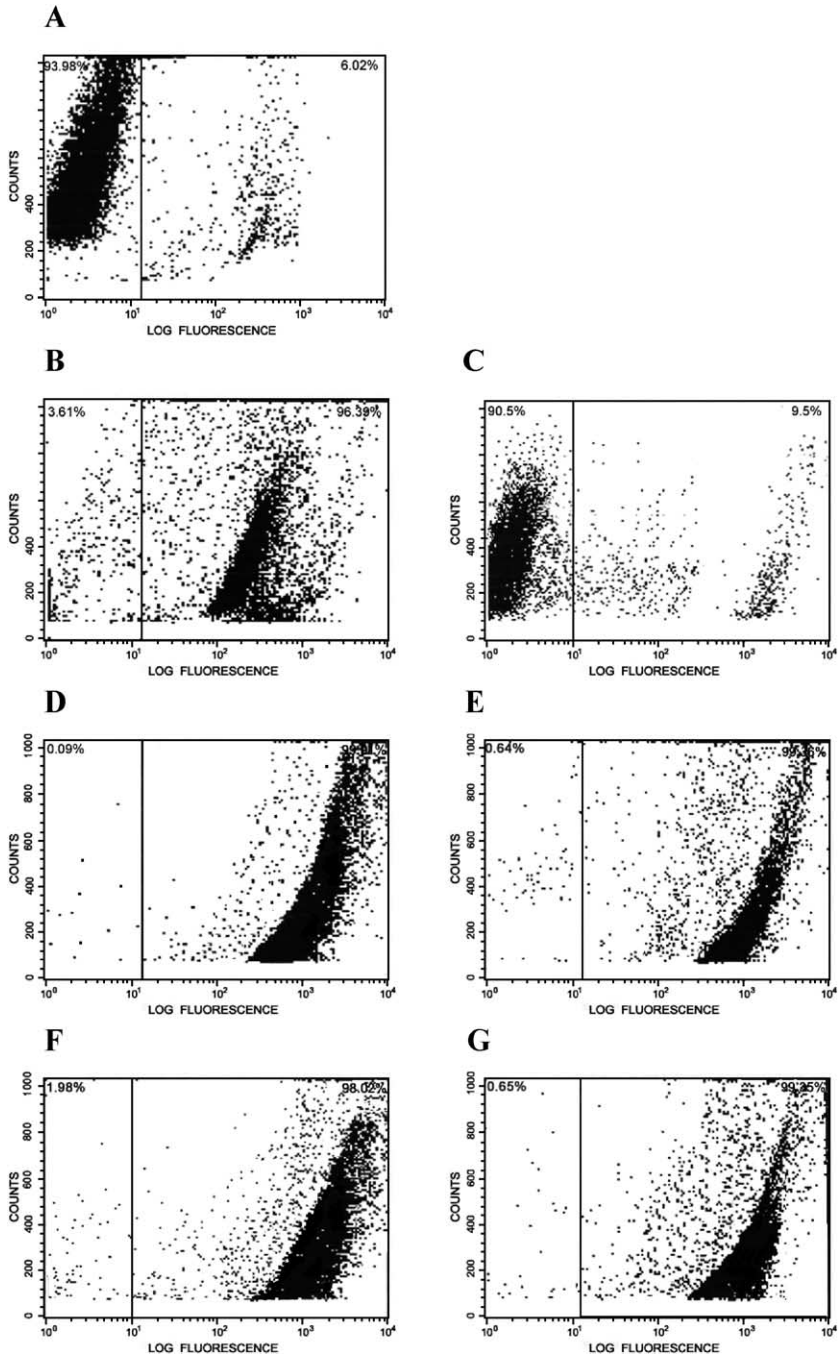

Fig. 5. The effect of NaN_3 on the CA–MA and P5. Exponential phase *C. albicans* cells were treated with 20 μM of the CA–MA and P5. The cell population showing fluorescence was analyzed by the FACScalibur flow cytometer. The increments of the log fluorescence signal represent PI uptake by peptides. (A) Control, (B) CA–MA-treated cells, (C) CA–MA- and NaN_3-treated cells, (D) P5-treated cells, (E) P5- and NaN_3-treated cells, (F) melittin-treated cells, (G) melittin- and NaN_3-treated cells.

by the nonviable cells, was highly dependent. In case of P5 or melittin, no energy dependency was observed indicating that the interaction does not require a cellular metabolic function. This result indicated that antifungal action of P5 is similar to that of melittin, which is membrane disruption [30].

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. The salt influences to the antifungal activity of synthetic peptides were investigated using a FACScalibur flow cytometer.

The antifungal activity of CA–MA was repressed by the 150-mM sodium chloride, in which the interaction of P5 or melittin with *C. albicans* cells was not salt dependent at the same concentration (Fig. 6).

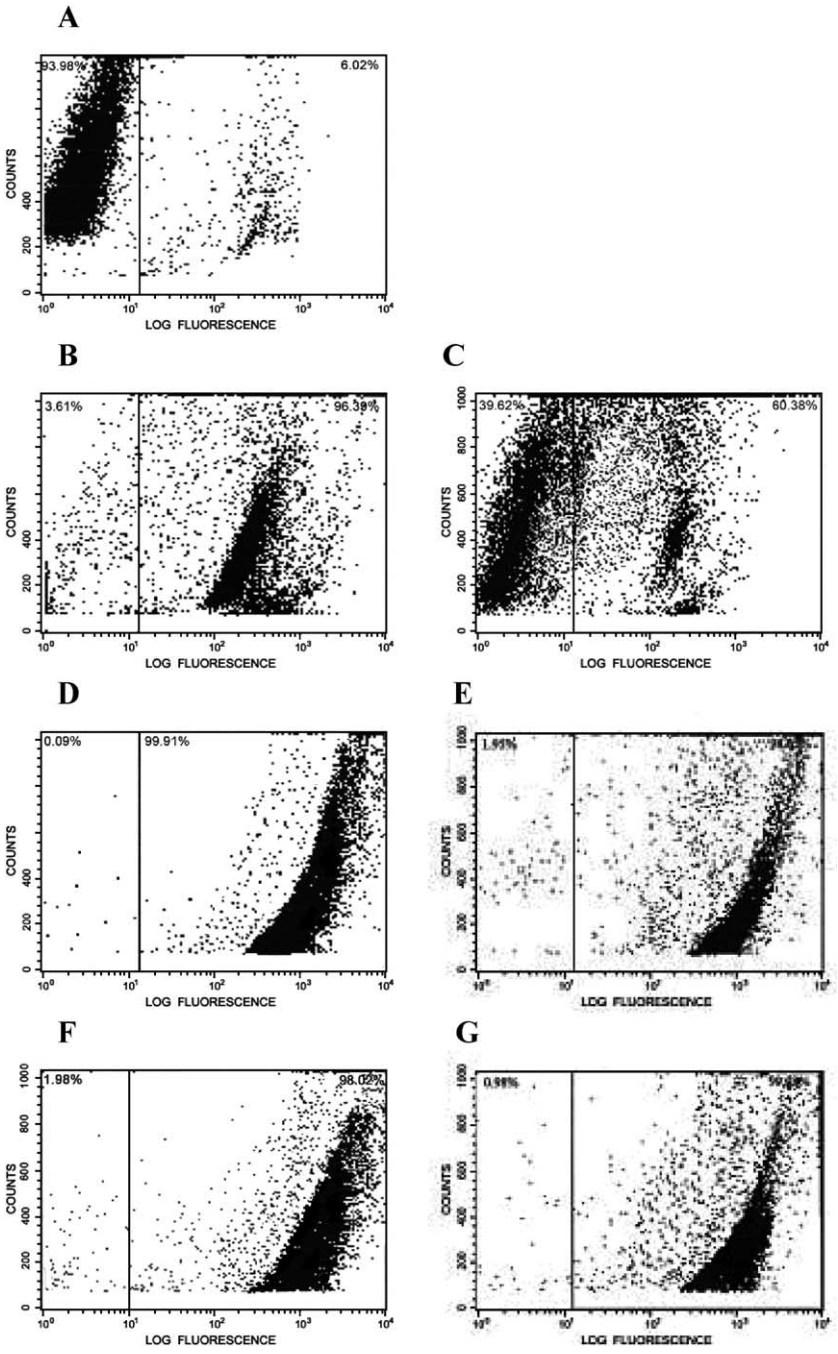

Fig. 6. Flow cytometric measurement of the effects of CA–MA and P5 with salt. The fluorescence of PI was monitored in the FL2-H channel. Flow cytometric analysis was performed by the FACScalibur flow cytometer. (A) Control, (B) CA–MA-treated cells, (C) CA–MA- and 150 mM NaCl-treated cells, (D) P5-treated cells, (E) P5- and 150 mM NaCl-treated cells, (F) melittin-treated cells, (G) melittin- and 150 mM NaCl-treated cells.

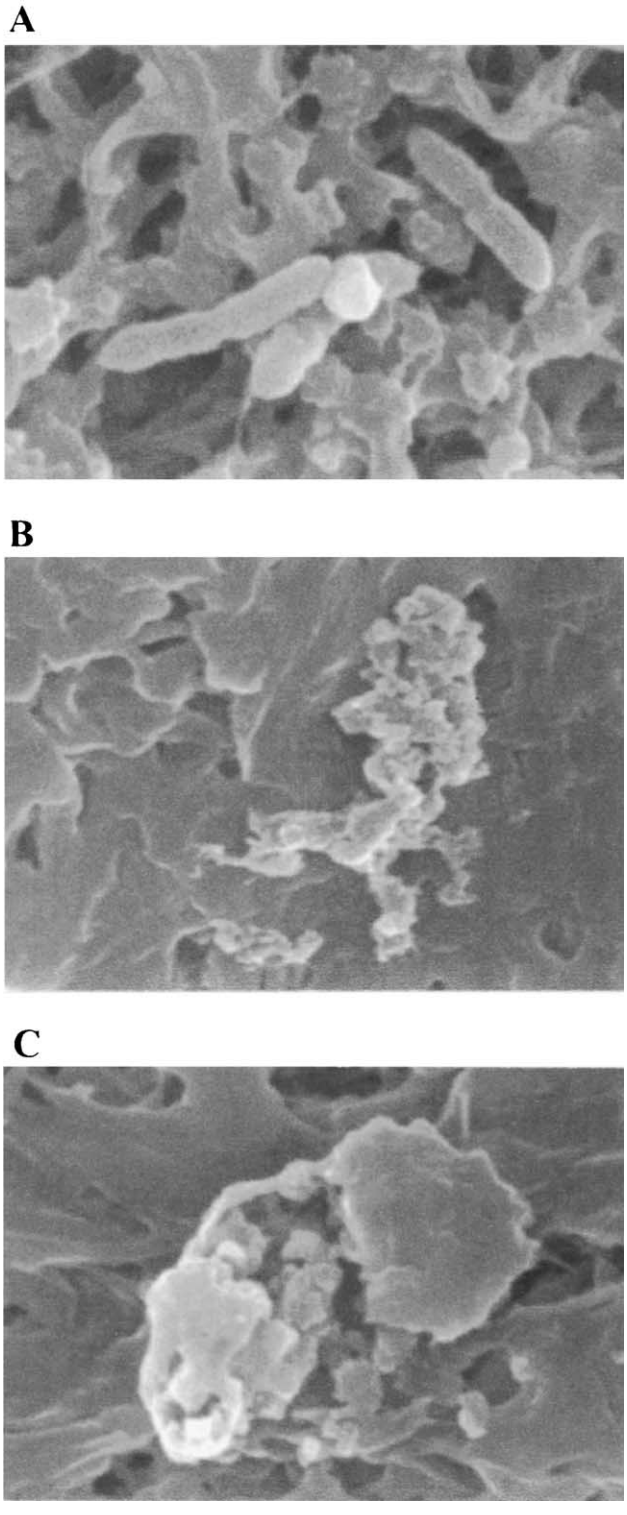

Fig. 7. Scanning electron micrographs of untreated (A) and after treatment for 30 min at 37 °C with 1.56 μM peptides of *P. aeruginosa*. Midlog phase *B. subtilis* or *P. aeruginosa* were resuspended at 10^8 CFU/ml in Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37 °C with CA–MA and P5. (A) No treated peptide; (B) P5; (C) melittin.

3.5. Analysis of the peptide under bacteria morphology and SUVs

We also examined morphological changes induced by the P5 on SEM. Untreated *P. aeruginosa* had a normal smooth surface (Fig. 7A). In contrast, cells treated for 4 h with P5 and melittin showed cell surface disruption (Fig. 7B and C, respectively). The SEM observations provide morphological evidence of the potent permeabilizing activity of the P5. The cell membrane alterations are similar to those induced by melittin used as a comparison.

To further confirm the ability of P5 to disrupt microbial plasma membrane, experiment was performed with liposome. Artificial SUVs (PC/cholesterol; 10:1, w/w) and neutral vesicles of PC were used as model membrane systems. In a recent report, the effect of the peptide has been proposed as two models, 'Barrel-stave model' [31] and 'Carpet model' [32]. The four steps proposed to be in the latter model are: (i) binding of peptide to phospholipid head groups; (ii) alignment of peptide on the surface of the membrane; (iii) rotation for reorientation of peptide toward membrane; (iv) disintegrating the membrane by disrupting the bilayer curvature. We cannot show pore on liposomes; we can show only the disruption of the membrane (Fig. 8). These results provide additional evidence that P5 probably acts on

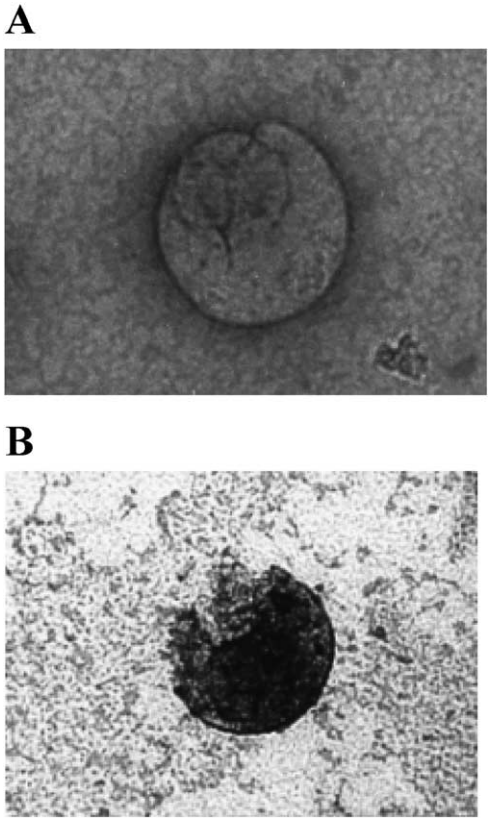

Fig. 8. Electron micrographs of negatively stained SUV composed of PC/cholesterol (10:1, w/w) in the absence (A), or in the presence (B), of P5. Specimens were examined in a TECNAI 12 (Philips) at an accelerating voltage of 120 kV. The scale bar represents 100 nm.

the plasma lipid membrane, by forming pores, causing the leakage of ions and other materials from the cells.

4. Conclusions

To obtain antibiotic peptides useful for pharmaceutical applications, a strong antibiotic activity is required against bacterial, fungal or cancer cells. In the present study, the several analogues were designed to increase net positive charge and hydrophobicity by Lys and Leu substitutions, Lys addition, flexible region (Gly-Ile-Gly → Pro) substitution or HSAKKF deletion. In particular, P5, flexible region (GIG → P) substitution, Lys (positions 4, 8, 14, 15) and Leu (positions 5, 6, 12, 13, 16, 17, 20) substitutions in CA–MA induced the enhanced antibiotic activity without hemolysis. This P5, which exhibits potent antimicrobial and antitumor activity, may have a potential as a specific pharmacological agent, as a model for the study of the net positive charge, hydrophobicity–antibiotic relationship of peptides, as well as in the development of a novel therapeutic agent.

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