Influences of Hinge Region of a Synthetic Antimicrobial Peptide, Cecropin A(1-13)-Melittin(1-13) Hybrid on Antibiotic Activity

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A synthetic cecropin A(1-13)-melittin(1-13) [CA-ME] hybrid peptide was known to be an antimicrobial peptide having strong antibacterial, antifungal and antitumor activity with minimal cytotoxic effect against human erythrocyte. Analogues were synthesized to investigate the influences of the flexible hinge region of CA-ME on the antibiotic activity. Antibiotic activity of the peptides was measured by the growth inhibition against bacterial, fungal and tumor cells and vesicle-aggregating or disrupting activity. The deletion of Gln-Gly-Ile (P1) or Gly-Gln-Gly-Ile-Gly (P3) from CA-ME brought about a significant decrease on the antibiotic activities. In contrast, Gly-Ile-Gly deletion (P2) from CA-ME or Pro insertion (P5) instead of Gly-Gln-Gly-Ile-Gly of CA-ME retained antibiotic activity. This result indicated that the flexible hinge or β-bend structure provided by Gly-Gln-Gly-Ile-Gly, Gln-Gly, or Pro in the central region of the peptides is requisite for its effective antibiotic activity and may facilitate easily the hydrophobic C-terminal region of the peptide to penetrate the lipid bilayers of the target cell membrane. In contrast, P4 and P6 with Gly-Gln-Gly-Pro-Gly or Gly-Gln-Pro in the central region of the peptide caused a drastic reduction on the antibiotic activities. This result suggested that the consecutive β-bend structure provided by Gly-Gln-Gly-Pro-Gly or Gly-Gln-Pro in the central hinge region of the peptide seems to interrupt the ion channel/pore formation on the target cell membranes.

Introduction

Cationic antimicrobial peptides have been found in a variety of sources, from prokaryotes to eukaryotes. In recent years, it has become clear that these endogeneous antimicrobial peptides constitute part of the host defense system and innate immunity.[1-3] The rapid emergence of antibiotic resistance has been of great concern in recent years.[4] Thus, there is great interest in the development of new classes of antibiotic agents.[5] Among the possible candidates, these endogeneous antimicrobial peptides have been attracted increasing clinical interest.[6,7] These cationic antimicrobial peptides were believed to act by forming amphipahtic α-helix which by interacting with cell membranes, lead to subsequent membrane disruption by means of ion channel/pore formation and eventually, cell death.[8,9] Cecropin A, a cationic 37-amino acid antimicrobial peptide, was initially isolated from the hemolmph of *Hyalophora cecropia* pupae.[10] Cecropin A is composed of two α-helices linked by a flexible hinge containing Gly-Pro residues. The N-terminal segments of cecropin A are strongly basic and form nearly perfect amphipathic α-helices, while the C-terminal portion consist of more hydrophobic helices. Cecropin A has a powerful lytic activity on bacterial cells and broad spectrum against Gram-positive and Gram-negative bacteria but possess no cytotoxic effects against human erythrocytes and other eukaryotic cells.[10,11] The bee venom toxin, melittin, is a cationic 26-amino acid antimicrobial peptide. Melittin shows a strong lytic action against bacterial cells, but has a strongly hemolytic activity and toxicity against eukaryotic cells.[12] It also consists of two α-helices linked a flexible segment.[12,13] In contrast to cecropin A, the N-terminal α-helix is hydrophobic and the C-terminal α-helix is basic. Its N-terminal hydrophobic segment was known to be responsible for the hemolytic activity of the molecule.

At first, the possible application of antimicrobial peptides requires the elimination of undesirable harmful effects such as hemolytic activity. In the course of the structure-antibiotic activity studies aimed at design an ideal antimicrobial peptides having improved antibiotic activity with no hemolytic activity, CA(1-13)-ME(1-13) [CA-ME], a 26-residue peptide containing parts of the N-terminal sequence of CA and ME has been found to have strong antibacterial and antitumor activity with minimal hemolytic activity.[14-16]

Cecropin A was predicted to have a helix-flexible hinge-helix motif from sequence analysis and CD measurements, and 2D-NMR studies.[17-19] CA-ME is composed of an N-terminal cationic amphipathic α-helix and a C-terminal hydrophobic α-helix joined by a flexible hinge region of Gln-Gly-Ile sequence. However, the effects of the flexible hinge region of this peptide on antibiotic activity against fungal and tumor cells as well as bacterial cells have not yet been investigated. Thus, in this present study, analogues were synthesized to investigate the influences of the flexible hinge region of CA-ME on the antibiotic activities. The peptides were synthesized by the solid phase method as C-terminal amides using N-9-fluorenylmethoxycarbonyl (Fmoc)-chem-

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istry.[20] In Table 1, the primary sequences of the chemically synthesized peptides used in this study are listed. Concentrations of the synthetic peptides were determined by amino acid analysis. Six bacterial and seven fungal strains and four tumor cells were used for antibacterial, antifungal and antitumor assays of the peptides. The membrane-aggregating and disrupting activities of the synthetic peptides were determined using the acidic phosphatidylcholine (PC)-phosphatidylserine (PS) (4 : 1, w/w) vesicles. Human erythrocyte and a mouse fibroblast cell line were used to measure the cytotoxicity of the peptides against normal eukaryotic cells. The secondary structures of the synthetic peptides in sodium dodecyl sulfate (SDS) solution, a membrane-mimetic environment were determined by circular dichroism (CD) spectra.

Experimental Section

Peptide synthesis and purification. Rink Amide 4-methylbenzhydrylamine (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 (Thr), trityl (Gln), *tert*-butyloxycarbonyl (Lys). Deprotection and cleavage from the resin were carried out using the mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol and triisopropylsilane (88 : 2.5 : 2.5 : 2.5 : 2.5 : 2.0, v/v) for 2 hr at room temperature. The crude peptide was then repeatedly washed with diethylether, and dried in vacuum. The crude peptides were purified by a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19×30 cm). Purity of the purified peptides 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 a vacuum. The residues were dissolved in 0.02 N HCl and subjected to amino acid analyzer (Hitachi Model, 8500 A, Japan). Peptide concentration was determined by amino acid analysis. The molecular weights of the synthetic peptides was determined using the matrix-assissted laser desorption ionization (MALDI) mass spectrometer.

Cytotoxicity against human erythrocyte. Human erythrocytes were centrifuged and washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer / 0.15 M NaCl, pH 7.0). One hundred μL of human erythrocytes suspended 0.8% (v/v) in PBS were plated into 96-well plates, and then 100 μL of the peptide solution was added to each wells. The plates were incubated for 1 hr at 37 °C, and centrifuged at 150 g for 5 min. One hundred μL 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 E_{max}, Sunnyvale, California). Zero percent hemolysis and 100% hemolysis were determined in PBS and 0.1% Triton-X 100, respectively. The hemolysis percentage was calculated using the equation:

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

Antibacterial activity. *Escherichia coli* (KCTC 1682), *Salmonella typhimurium* (KCTC 1926), *Pseudomonas aeruginosa* (KCTC 1637), *Bacillus subtilis* (KCTC 1918), *Streptococcus pyogenes* (KCTC 3096), *Staphylococcus aureus* (KCTC 1621) were supplied from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Taejon, Korea). The bacteria were grown to the mid-logarithmic phase in Luria-Bertani medium (10 g of Bacto-tryptone of per liter, 5 g of Bacto-yeast extract per liter [both from Difco Laboratories] and 10 g NaCl per liter). The peptides were sterilized 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 formation units (CFU)/mL) suspended in growth medium (100 μL) was mixed with 100 μL of the test peptide solution in a microtiter plate well with three replicates for each test solution. Microbial growth was determined by the increase in optical density at 620 nm after 10 h incubation at 37 °C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no change in optical density.

Kinetics of bacterial killing. The kinetics of bacterial killing of the peptides were evaluated using *E. coli* and *B. subtilis*. Log-phase bacteria (6×10^5 CFU/mL) were incubated with 1.0 μM peptide in LB broth. Aliquots were removed at fixed time intervals, appropriately diluted, plated on LB broth agar plate, and then the colony-forming units were counted after 16-18 h incubation at 37 °C.

Antifungal activity. *Aspergillus flavus* (KCTC 1375), *Aspergillus fumigatus* (KCTC 6145), *Aspergillus niger* (KCTC 2025), *Saccharomyces cerevisiae* (KCTC 2805), *Trichoderma viridae* (KCTC 6047), *Trichosporon beigelii* (KCTC 7251), and *Candida albicans* (KCTC 1940) were supplied from KCTC, KRIBB (Taejon, Korea). The fungal strains were grown at 28 °C in a YM medium (1% glucose, 0.3% malt extract, 0.5% peptone, and 0.3% yeast extract). The fungal conidia were seeded on 96-well microtiter plates at a density of 1×10^2 spores per well in a volume of 100 μL of YM media. 10 μL of the serially diluted-peptides was added to each well, and the cell suspension was incubated for 24 hr at 28 °C. After incubation, 10 μL of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-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 further incubated at 37 °C for 4 hr. Forty μL of 20% SDS solution containing 0.02 M HCl was added and the reaction incubated at 37 °C for 16 hr to dissolve the formed MTT-formazan crystals. The absorbance of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices, Sunnyvale, California).

Antitumor activity. Growth inhibitory activity of the peptides against cancer and normal fibroblast cells was deter-

mined as 50% inhibition concentration (IC_{50}) using a tetrazolium (MTT) assay. Human chronic myelogenous leukemia (K-562: ATCC cat no. CCL-243), human acute T cell leukemia (Jurkat: ATCC cat no. TIB-152), human lung carcinoma cancer cells (K-549: ATCC cat no. CCL-185) and human breast adenocarcinoma cell (MDA-MB-361: ATCC cat no. HTB-27) were used for the growth inhibitory activity assay of the peptides against cancer cells. Mouse NIH-3T3 fibroblast cell (ATCC cat no. CRL-1658) were used for growth inhibitory activity assay of the peptides against normal cells. These cells were obtained from the Genetic Resources Center of Korea Research Institute of Bioscience & Biotechnology (Taejon, Korea). The cells were grown in a RPMI-1640 medium 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 were added and then incubated for 3 days. Twenty μL of MTT solution was added to each well and the plates were incubated at 37 °C for 4 h. Forty μL of 20% SDS solution containing 0.02 M HCl was added in each well, and then incubated 37 °C for 3 hr. Absorbance was measured at 570 nm on an ELISA plate reader (Molecular Devices E_{max}, California, USA).

Vesicle-aggregating activity. Phospholipid vesicle aggregating activity of the peptides was measured by the changes in turbidity at 400 nm using an Beckman DU-8 spectrophotometer (Pamlo Alto, CA, USA). Phospholipid vesicle suspension composed of phosphatidylcholine (PC)/ phosphatidylserine (PS) (4 : 1, w/w) was prepared in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) to give a final concentration of 35 μM by the sonication process.[21] Various amounts of the peptides were added to the vesicle suspension to obtain various ratios of peptide to phospholipid.

Vesicle-disrupting activity. Carboxyfluorescein (CF)-encapsulated large unilamellar vesicles (LUV) composed of PC/PS (4 : 1, w/w) were prepared by the reversed-phase ether evaporation method using 100 mM CF.[22] The initially formed vesicles were extruded through Nucleopore filter of 0.1 μm. To remove free CF dye, the vesicles were passed through a Bio-Gel A 0.5 m (Bio-Rad, Richmond, USA) column (1.5×30 cm) using phosphate buffered saline, pH 7.4, as the eluting buffer. The separated LUV fraction, after appropriate dilution to a final concentration of 6.36 μM, was mixed with the peptide solution in a 2 mL cuvette at 25 °C. The leakage of CF from the LUV was monitored by measuring fluorescence intensity at 520 nm excited at 490 nm on a Shimazu RF-5000 spectrofluorometer (Tokyo, Japan). The apparent percent leakage value at a fluorescence intensity, F, was calculated by the following equation:

$$\% \text{ leakage (apparent)} = 100 \times (F - F_0) / (F_t - F_0)$$

F_t denotes the fluorescence intensity corresponding to 100% leakage after the addition of 20 μL of 10% Triton X-100. F_0 represents the fluorescence of the intact vesicle.

Circular dichroism (CD) spectroscopy. CD spectra of peptides were recorded using a Jasco J720 spectropolarimeter using a 1 mm pathlength cell (Japan). The CD spectra of the peptides in the presence of 30 mM SDS in 10 mM sodium phosphate buffer (pH 7.2) were recorded at 25 °C in the 190-240 nm wavelength range at 0.1 nm intervals. The peptide concentrations were 100 μg/mL. The mean residue ellipticity [θ] was calculated using the molecular weight of each peptide as determined from the amino acid composition. The percentage helicity of the peptides were calculated with the equation:[23]

$$\% \text{ helix} = 100 ([\theta]_{222} - [\theta]^0_{222}) / [\theta]^{100}_{222},$$

where $[\theta]_{222}$ is the observed mean residue ellipticity per residue at 222 nm in deg · cm^2 · dmol^{-1}. $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$ are the estimated ellipticities corresponding to a random coil (–3,000 deg · cm^2 · dmol^{-1}) and 100% helical peptides (–33,000 deg · cm^2 · dmol^{-1}), respectively.

Results and Discussion

A hybrid peptide, CA-ME composed of the N-terminal region of cecropin A and melittin has been reported to have more potent antibacterial activity with minimal hemolytic activity than parental cecropin A.[14,15] Also, in our previous study, CA-ME showed powerful lytic activity against human small cell lung cancer (SCLC) cells, fungal cells and acidic liposomes composed of PC and PS as well as bacterial cells.[16] Ehrenberg *et al.* reported that the Gln-Gly-Ile hinge sequence of in the central region between two well-defined α-helical regions of CA-ME in the hydrophobic environment was observed to be very flexible by two-dimensional NMR study.[24] In this study, analogues (Table 1) were synthesized to investigate the influences of the flexible hinge

Table 1. Amino acid sequences of CA-ME and its analogues

Peptides	Amino acid sequences	Remarks
CA-ME	KWKLFKKIEKVGQGIGAVLKVLTTGL-NH_2	CA(1-13)-ME(1-13)
P1	KWKLFKKIEKVG-----GAVLKVLTTGL-NH_2	CA(1-13)-ME(1-13): deletion (QGI)
P2	KWKLFKKIEKVGQ-----AVLKVLTTGL-NH_2	CA(1-13)-ME(1-13): deletion (GIG)
P3	KWKLFKKIEKV---------AVLKVLTTGL-NH_2	CA(1-13)-ME(1-13): deletion (GQGIG)
P4	KWKLFKKIEKVGQP---AVLKVLTTGL-NH_2	CA(1-13)-ME(1-13): GIG → P
P5	KWKLFKKIEKV----P----AVLKVLTTGL-NH_2	CA(1-13)-ME(1-13): GQGIG → P
P6	KWKLFKKIEKVGQGPGAVLKVLTTGL-NH_2	CA(1-13)-ME(1-13): I^{15} → P

Table 2. Molecular weights of the peptides determined by MALDI-MS

Peptides	Observed value	Calculated value
CA-ME	2854.39	2853.86
P1	2557.28	2556.16
P2	2628.49	2627.14
P3	2441.10	2441.96
P4	2723.15	2724.25
P5	2538.67	2539.07
P6	2838.95	2838.35

sequence in CA-ME in its antibacterial, antifungal, antitumor, hemolytic and vesicle-aggregating or disrupting activities.

The purity and amino acid composition of all synthetic peptides determined by amino acid analysis (data not shown). The correct molecular weights of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 2). Antibacterial and antifungal activities of the synthetic peptides for six bacterial and seven fungal cells were determined as the minimal inhibitory concentration (MIC) and 50% inhibition concentration (IC_{50}) by the microdilution test in 96-well plates, respectively (Table 3 and 4). Antitumor activity of the peptides against the four different tumor cells, K-562, Jurkat, A-549 and MDA-MB-361 were determined as the IC_{50} by the tetrazolium (MTT) assay (Table 5).

The deletion (P1) of Gln-Gly-Ile or Gly-Gln-Gly-Ile-Gly (P3) from CA-ME caused a remarkable reduction in antibacterial, antifungal, antitumor and hemolytic activities. The deletion (P2) of Gly-Ile-Gly sequence from CA-ME and Pro insertion instead of Gly-Gln-Gly-Ile-Gly in CA-ME retained the antibiotic activity containing antibacterial, antifungal, antitumor activity. This result indicated that the flexible hinge or β-bend structure provided by Gly-Gln-Gly-Ile-Gly, Gln-Gly, or Pro of the peptide is requisite for its effective antibiotic activity and may facilitate easily the hydrophobic C-terminal region of CA-ME to penetrate the lipid bilayers

Table 3. Antibacterial activities of CA-ME and its analogues

Peptides	Gram-negative bacteria : MIC (μM)			Gram-positive bacteria: MIC (μM)		
	E. coli	*S. typhimurium*	*P. aeruginosa*	*B. subtilis*	*S. pyogenes*	*S. aureus*
CA-ME	1.56	1.56	0.78-1.56	1.56	1.56	1.56
P1	12.5	3.125	3.125	12.5	12.5	25.0
P2	1.56	1.56-3.125	0.78	1.56	1.56	1.56
P3	12.5	6.25	3.125	12.5	12.5	12.5
P4	6.25	1.56	0.78	6.25	6.125	12.5-25.0
P5	1.56	0.78	0.39-0.78	1.56-3.125	1.56	3.125
P6	12.5	3.125	1.56	6.25-12.5	6.25	12.5

Table 4. Antifungal activities of CA-ME and its analogues against filamentous fungi and yeast strains

Peptides	Filamentous fungi: IC_{50} (μM)				Yeast strains IC_{50} (μM)		
	A. flavus	*A. fumigatus*	*A. niger*	*T. viridae*	*S. cerevisiae*	*T. beigelii*	*C. albicans*
CA-ME	10.0	10.2	8.0	13.2	11.8	8.9	9.8
P1	71.0	69.0	80.0	55.9	73.2	62.1	50.1
P2	8.0	7.1	7.0	11.3	9.8	8.0	10.3
P3	61.0	63.0	81.0	59.1	75.2	63.8	55.5
P4	50.0	49.0	53.0	49.2	65.2	53.8	46.6
P5	9.0	9.3	7.1	12.4	10.2	9.0	9.5
P6	41.0	39.0	49.0	39.5	43.2	41.8	50.0

Table 5. Antitumor and hemolytic activities of CA-ME and its analogues

Peptides	Tumor cells (IC_{50}: μM)				Fibroblast cell (NIH-3T3) (IC_{50}: μM)	% Hemolysis (100 μM)
	K-562	A-549	Jurkat	MDA-MB 361	NIH-3T3	Erythrocyte
CA-ME	14.0	8.0	5.0	18.0	50.0	14.0
P1	> 100	> 100	> 100	> 100	> 100	0
P2	6.0	6.0	3.1	16.0	60.0	11.6
P3	25.0	60.0	60.0	34.0	100	27.2
P4	> 100	100	> 100	> 100	> 100	0
P5	50	40.0	22.0	42.0	100	0.7
P6	100	>100	> 100	> 100	> 100	0

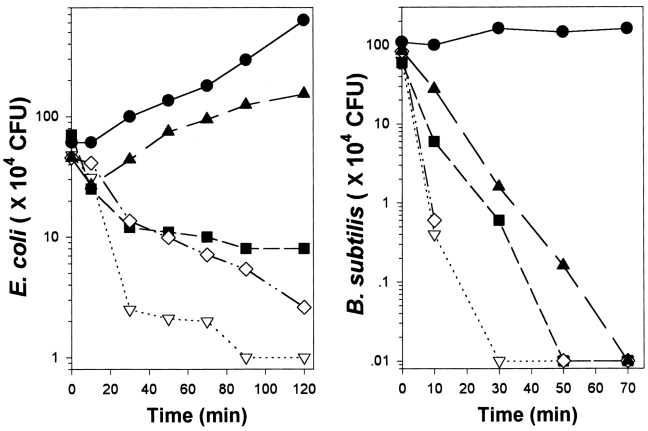

Figure 1. Kinetics of killing *E. coli* and *B. subtilis* by CA-ME and its analogues. Bacteria, either untreated (●) or treated with 1.0 μM CA-ME (▽), 1.0 μM P1 (■), 1.0 μM P2 (◇), or 1.0 μM P6 (▲), were diluted at the indicated time intervals, and then plated on LB broth agar. The colony forming units were calculated by counting the plates after 16-18 hr incubation at 37 °C.

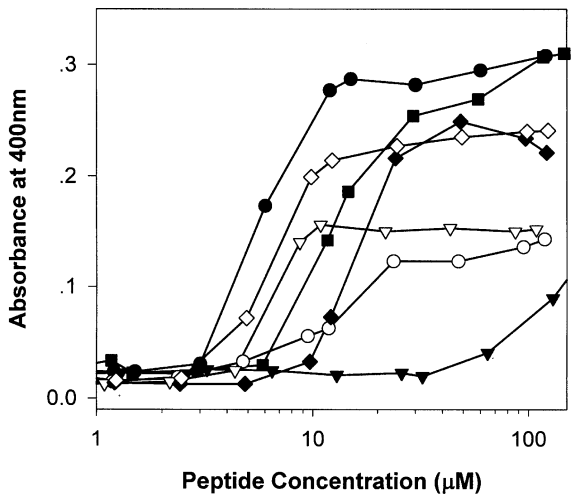

Figure 2. Monitoring the phospholipid vesicle (PC/PS, 4 : 1) aggregation induced by CA-ME (●), P1 (▼), P2 (▽), P3 (■), P4 (◆), P5 (◇) and P6 (○). The vesicle aggregation was determined by measuring the absorbance at 400 nm.

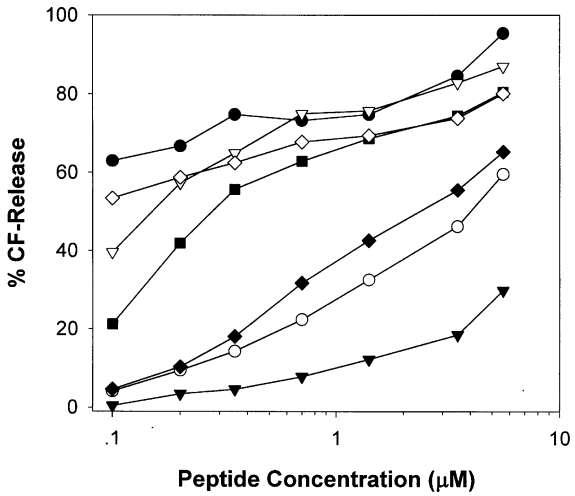

Figure 3. CF-Release from LUVs composed of PC : PS (4 : 1) induced by by CA-ME (●), P1 (▼), P2 (▽), P3 (■), P4 (◆), P5 (◇) and P6 (○). The released CF fluorescence was measured at $\lambda_{excitation}$ = 490 nm and $\lambda_{emission}$ = 520 nm.

of the target cell membrane. In contrast, P6 and P4 with Gly-Gln-Gly-Pro-Gly or Gly-Gln-Pro in the central region of the peptide caused a significant decrease on the lytic activity against *E. coli*, three Gram-positive strains tested, fungal and tumor cells and human erythrocytes. This result suggested that the consecutive β-bend structure provided by Gly-Gln-Gly-Pro-Gly or Gly-Gln-Pro in the central hinge region of the peptide seems to interrupt the ion channel/pore formation on the target cell membranes.

Also, the time course of the synthetic peptides to kill mid-logarithmic phase of *E. coli* and *B. subtilis* were compared. As shown in Figure 1, CA-ME and P2 displayed faster bactericidal rate against both *E. coli* and *B. subtilis* than P1 and P6. These results indicate that the flexibility provided by Gly-Gln-Gly-Ile-Gly or Gly-Gln sequence in the central region of the peptide have an important role in the bactericidal rate as well as bactericidal activity against both *E. coli* and *B. subtilis*.

To improve the therapeutic index of toxic peptides, antibiotic activity with minimal cytotoxicity toward host cells is needed. As an indication for this index, the lytic activity of the peptides against normal NIH-3T3 fibroblast cells was compared. All peptides were less effective against normal fibroblast cells than malignant cells (Table 5). This result may be due to the outer membrane surface of malignant cells having more exposed anionic phospholipids such as phosphatidylserine than normal cells.[25] This interpretation was supported by Chen *et al.* study.[26] Cecropin B analogue with more positive charged residues than cecropin B was more effective against HL-60, K-562, Jurkat and CCRF-CEB leukemia cancer cells as compared with cecropin B.

To investigate cytoplasmic membrane disruption induced by cationic peptides, peptide-phospholipid interactions were examined in phospholipid vesicles composed of PC/PS (4 : 1) as a model membrane system. The vesicle aggregation event was regarded as the initial step in peptide-vesicle interaction. The changes in vesicle size due to vesicle aggregation could be monitored by the absorbance increase at 400 nm. The light scattering is known to be quite sensitive to particle size. The vesicle aggregation efficiency was determined at a function of the added peptide concentration to the vesicle (Figure 2). The rank order of vesicle aggregation induced by the peptides was CA-ME > P2, P5 > P3 > P1, P4, P6. The interaction of peptides with phospholipid vesicles was further investigated by measuring the fluorescence intensity of the released CF from LUV through the disruption of the vesicles (Figure 3). As shown in Figure 3, the phospholipid vesicles perturbation activity induced by the peptides was CA-ME > P2, P5 > P3 > P1, P4, P6. This result suggested that the relative efficiency to perturb the lipid membranes are correlated with their vesicle aggregation activities and the flexibile hinge or β-bend structure provided by Gly-Gln-Gly-Ile-Gly, Gln-Gly or Pro in the cetntral

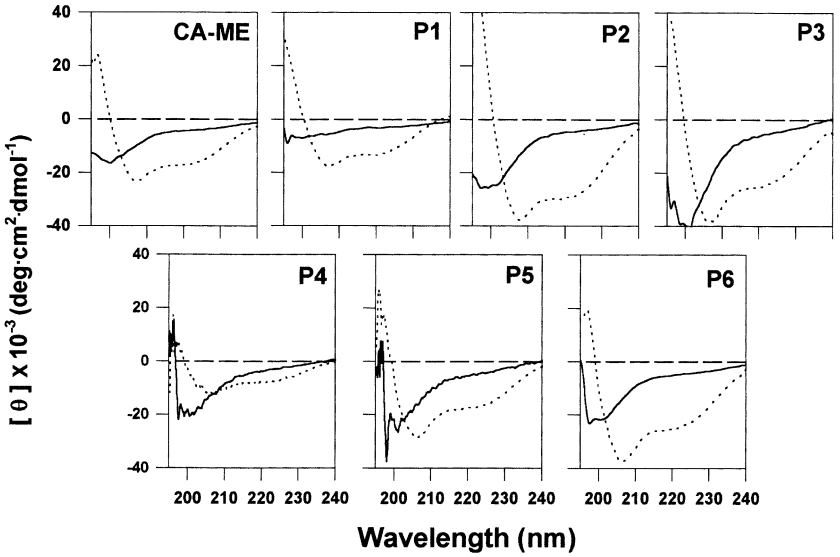

Figure 4. CD spectra of CA-ME and its analogues in the presence of 0 mM SDS (—), and 30 mM SDS (-----) in 10 mM sodium phosphate buffer, pH 7.2, respectively.

region of the peptide is important for the phospholipid interaction as well as antibiotic effect containing antibacterial, antifungal and antitumor activity. The vesicle disrupting activities were dependent on the peptide concentration in the vesicle suspension. Therefore the antibiotic activity of these peptides was due to the cytoplasmic membrane disruption through the peptide-lipid interaction. In peptide-phospholipid vesicle interaction studies, these peptides induced not only the aggregation but also the perturbation of vesicles in a dose-dependent manner. The importance of electrostatic interactions in the peptide-lipid interactions in the peptide-lipid vesicle was suggested by the results that these peptides specifically induced the aggregation of the vesicle containing negatively-charged phospholipid and the reduced aggregation efficiency by the addition of zwitterionic phosphatidylcholine to phosphatidylserine vesicle (data not shown). When the peptide was added to phospholipid vesicle solution, the positively charged N-terminal region of the peptide was electrostatically attracted to the negatively charged head group of acidic phospholipid and the flexible character of central hinge region helped the hydrophobic C-terminal region to penetrate the nonpolar tail of lipid bilayer.

In order to investigate the relationship of antibiotic activity and α-helix content of the peptides on cell membranes, the CD spectra of the peptides in the presence of 30 mM SDS micelles which mimics the cell membrane environments was measured. All peptides showed a random coil structure in aqueous buffer, while adopted the typical α-helical spectrum with two minimum peaks at 208 and 222 nm in 30 mM SDS solution. As shown in Figure 4 and Table 6, P1 and P4 adopt lower α-helical conformation than the other peptides. P1 and P4 resulted in a significant reduction in antibacterial, antifungal, antitumor, hemolytic and vesicle-aggregating or disrupting activities. In contrast, P6 with higher α-helix content than CA-ME displayed low lytic activities in antibacterial, antifungal, antitumor, hemolytic and vesicle-aggregating or disrupting activities as compared with CA-ME. These results suggested that the α-helical content of the peptides may not be correlated to their antibiotic activity.

Table 6. Percent -helicity of CA-ME and its analogues in SDS solution

Peptides	Buffer		30 mM SDS	
	$[\theta]_{222}$	α-helix (%)	$[\theta]_{222}$	α-helix (%)
CA-ME	-4,143.8	3.4	-16,773.4	41.7
P1	-3,054.2	0.4	-12,700.5	29.4
P2	-4,633.3	4.9	-28,944.1	78.6
P3	-5,371.0	7.2	-24,394.6	64.8
P4	-3,141.7	0.4	-7,596.5	13.9
P5	-4,918.9	5.8	-17,140.7	42.9
P6	-4,709.4	5.2	-24,582.0	65.4

Our results in this study will provide an important information about the structure-antibiotic activity relationships and the antibiotic mechanism of the antimicrobial peptides containing the central hinge sequence of the molecule.

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