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Analogues of Hybrid Antimicrobial Peptide, CAMA-P2, Designed with Improved Antimicrobial and Synergistic Activities

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We have designed a 20-residue hybrid peptide CA(1-8)-MA(1-12) (CAMA) incorporating residues 1-8 of cecropin A (CA) and residues 1-12 of magainin 2 (MA) with high bacterial cell selectivity. CAMA-P2 is an α-helical antimicrobial peptide designed from a CAMA hybrid peptide and substitution of Gly-Ile-Gly hinge sequence of CAMA to Pro influences the flexibility at central part of CAMA. Based on structure-activity relationships of CAMA peptides, to investigate the effects of the total positive charges on antimicrobial activity of CAMA-P2, the $Ser^{14} \rightarrow Lys$ analogue (CAMA-syn1) was synthesized. The role of tryptophan at C-terminal α-helix on its antimicrobial activity as well as synergistic activity was also investigated using $Ser^{14} \rightarrow Lys$/$Phe^{18} \rightarrow Trp$ analogue (CAMA-syn2). Also, we designed CAMA-syn3 by substitution of Lys^{16} located opposite side of substituted Lys^{14} of CAMA-syn1 with Leu residue, resulting in increase of hydrophobicity and amphipathicity of the peptide. All of CAMA-syn analogues showed good antimicrobial activities similar to those of CAMA and CAMA-P2. The CAMA-syn1 and CAMA-syn2 showed low hemolytic activity and cytotoxicity against human keratinocyte Haca-T cells while CAMA-syn3 showed hemolytic activity and cytotoxicity at its MIC value. We then investigated their abilities to act synergistically in combination with the antimicrobial flavonoids and synthetic compounds screened in our laboratory. The results showed that all peptides exhibited synergistic effects with dihydrobinetin, while only CAMA-syn2 exhibited synergistic effects with YKAs3001 against both *S. aureus* and MRSA, suggesting that Trp residue at C-terminus of CAMA-syn2 may facilitate the polar antibiotic flavonoids and synthetic compounds to permeabilize the membrane. This study will be useful for the development of new antibiotic peptides with potent antimicrobial and synergistic activity but without cytotoxicity.

Key Words : CAMA analogues, Antimicrobial activity, Structure-activity relationship, Flavonoid, Synergistic effect

Introduction

Antimicrobial peptides are an abundant and diverse group of molecules that are produced by many tissues and cell types in a variety of invertebrate, plant and animal species.[1-6] These natural antimicrobial peptides are known to play important roles in the host defense system and innate immunity.[1-5] Since antibiotic resistance develops rapidly as soon as new antimicrobial agents are introduced, attempts to develop antimicrobial compounds with novel mechanisms of action resulted in increased interest in the mode of action of antimicrobial peptide.[7-12] Cecropin A (CA) is a 37 residue antimicrobial peptide isolated from the hemolymph of the giant silk moth, *Hyalophora cecropia*.[3,8,9,13,14] Magainin 2 (MA) is a 23 residue antimicrobial peptide discovered in the skin of the African clawed frog, *Xenopus laevis*.[4,15] CA and MA display powerful lytic activity against both Gram-positive and Gram-negative bacteria without cytotoxic effects against human erythrocytes and other eukaryotic cells. These cationic antimicrobial peptides form amphipathic α-helix, which leads to subsequent membrane disruption by means of ion channel/pore formation, and eventually cell death.[4,16]

In the course of systematic studies aiming of finding potent antibiotic peptides with improved antimicrobial activity without hemolytic effect, a series of hybrid peptides have been developed.[16-20] For example, cecropin A-magainin 2 (CAMA) hybrid peptides comprising the N-terminal amphipathic basic region of CA and the N-terminal hydrophobic region of MA and cecropin A-melittin (CAME) hybrid peptides comprising the N-terminal amphipathic basic region of CA and the N-terminal hydrophobic region of melittin (ME) displayed high antimicrobial and antitumor activities, but showed no hemolytic activity at 100 μg/mL.[16-22]

We have designed CAMA-P2 (KWKLFKKIPKFLHSA-KKF-NH_2), an α-helical antimicrobial peptide analogue of a CAMA hybrid.[22] Substitution of Gly-Ile-Gly sequences of CAMA to Pro influence the flexibility at central part of CAMA and is important in antimicrobial and pore-forming activity to allow the attachment to and insertion into cell membrane.[22] We also found that Trp residue at the N-terminus of CAMA lead to the primary binding to the cell membrane.[22]

Recent reports have demonstrated that in vitro interaction of some cationic peptides with several clinically used antibiotics against several clinical isolates of Gram-positive and

Gram negative bacteria.[23-26] The use of combinations of antibiotics is common in the clinical setting and expands the spectrum of organism that can be targeted, prevents the emergence of resistant organism, decreases toxicity by allowing lower doses of both agents and can result in synergistic inhibition.[27] Synergistic effect was observed in several clinically isolated bacterial strains when some α-helical antimicrobial peptides were combined with several clinically used antibiotics.[28] Therefore, the synergistic effect with clinically used antimicrobial agents against antibiotic-resistant bacterial strains makes the cationic antimicrobial peptides potentially valuable as an adjuvant for antimicrobial chemotherapy. We have shown the synergistic effect of α-Helical Antimicrobial Peptide P18 with vancomycin, too.[29]

Here, based on structure-activity relationships of CAMA peptides, we designed new potent peptides with synergistic activity. We synthesized the hybrid peptide analogue CAMA-syn1 with substitutions of Ser^{14} with Lys in CAMA-P2, resulting in increase of total positive charge. Tryptophan residues are known to have important roles in the interactions between a peptide and a biological membrane. To investigate the role of Trp residue at C-terminus on its antimicrobial and synergistic activity, CAMA-syn2 analogue, in which the Phe^{18} residue in CAMA-syn1 is replaced with Trp residue was synthesized. Also, we designed CAMA-syn3 by substitution of Lys^{16} located opposite side of substituted Lys^{14} of CAMA-syn1 with Leu residue, resulting in increase of hydrophobicity and amphipathcity of the peptide. We evaluated their antimicrobial activities as well as toxicities to human erythrocytes. We then investigated their abilities to act synergistically in combination with polar antimicrobial flavonoids and synthetic compounds.

Methods

Peptide Synthesis. All peptides specified in Table 1 were prepared by solid-phase synthesis using Fmoc chemistry. Peptides were purified by reversed-phase preparative high-performance liquid chromatography on a C_{18} column (20 × 250 mm; Shim-pack) using a gradient of 20% to 50% acetonitrile in H_2O with 0.1% TFA delivered over 30 min.[30] Analytical high-performance liquid chromatography with an ODS column (4.6 × 250 mm; Shim-pack) revealed that purified peptides were more than 95% homogeneous (data not shown). The peptides also had the correct atomic masses as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Antimicrobial Activity. *Staphylococcus aureus* KCTC 1621 was purchased from the Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology (Taejon, Korea). Methicillin-resistant *Staphylococcus aureus* (MRSA) (CCARM 3089) was obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University in Korea. Antimicrobial activities of the peptides against bacteria were determined using a broth microdilution assay. Briefly, single colony of bacteria was inoculated into LB and cultured overnight at 37 °C. An aliquot of the culture was transferred to 10 mL fresh LB and incubated for an additional 3-5 h at 37 °C until mid-logarithmic phase. Two-fold serial dilutions of peptides in 1% peptone were prepared. Diluted peptides (100 μL) were added to 100 μL cells (2×10^6 CFU/mL) in 96-well microtiter plates and incubated at 37 °C for 16 h. The lowest concentration of peptide that completely inhibited growth was defined as the minimal inhibitory concentration (MIC). MIC values were calculated as the average from triplicate measurements in three independent assays.

Hemolytic Activity. Hemolytic activities of the peptides were tested against human red blood cells (h-RBC). Fresh h-RBCs were washed three times with phosphate-buffered saline PBS (PBS; 35 mM phosphate buffer containing 150 mM NaCl, pH 7.4) by centrifugation for 10 min at 1000 g and resuspended in PBS. The peptide solutions were then added to 50 μL of h-RBC in PBS to give a final volume of 100 μL and a final erythrocyte concentration of 4%, v/v. The resulting suspension was incubated with agitation for 1 h at 37 °C. The samples were centrifuged at 1000 g for 5 min. Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 405 nm. Controls for no hemolysis (blank) and 100% hemolysis consisted of human red blood cells suspended in PBS and 0.1% Triton X-100, respectively. The percent hemolysis was calculated using the following equation:

$$\text{Hemolysis (\%)} = [(OD_{405\ nm}\ \text{sample} - OD_{405\ nm}\ \text{zero lysis}) / (OD_{405\ nm}\ 100\%\ \text{lysis} - OD_{405\ nm}\ \text{zero lysis})] \times 100.[31]$$

Cytotoxicity Against Haca-T Cells. The human keratinocyte Haca-T cells were obtained from the Korea Research Institute of Chemical Technology (KRICT) (Daejon, Korea). Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum and antibiotic-antimyotic solution (100

Table 1. Amino acid sequences of CAMA, CAMA-P2 and CAMA-syn analogues and their hydrophobicities

Peptide	Sequences	M.W.	Net charge[a]	Hydrophobicity[b]
CAMA	KWKLFKKIGIGKFLHSAKKF-NH_2	2405.02	8.0	-0.34
CAMA-P2	KWKLFKKI**P**KFLHSAKKF-NH_2	2273.29	8.0	-0.60
CAMA-syn1	KWKLFKKI**P**KFLH**K**AKK**F**-NH_2	2314.47	9.0	-0.91
CAMA-syn2	KWKLFKKI**P**KFLH**K**AKK**W**-NH_2	2353.49	9.0	-0.93
CAMA-syn3	KWKLFKKI**P**KFLH**K**A**L**KF-NH_2	2299.33	8.0	0.17

[a]Net charge was calculated using sum of each of the amino acid charge at pH 7.0. [b]The mean hydrophobicity calculated by CCS scale is the total hydrophobicity (sum of all residue hydrophobicity indices) divided by the number of residues.[48]

units/mL penicillin, 100 mg/mL streptomycin and 25 mg amphotericin B) in 5% CO_2 at 37 °C.[32] Cultures were passed every 3 to 5 days, and cells were detached by brief trypsin treatment, and visualized with an inverted microscope. Cytotoxicities of peptides against Haca-T cells were determined using the MTT assay as reported previously.[33] Cells were seeded on 96-well microplates at a density of 2×10^4 cells/well in 150 μL RPMI1640 containing 10% fetal bovine serum. Plates were incubated for 24 h at 37 °C in 5% CO_2. Peptide solutions (20 μL) (serial 2-fold dilutions in RPMI1640) were added, and the plates further incubated for 1 day. Wells containing cells without peptides served as controls. Subsequently, 20 μL MTT solution (5 mg/mL) was added in each well, and the plates were incubated for a further 4 h at 37 °C. Precipitated MTT formazan was dissolved in 40 μL of 20% (w/v) SDS containing 0.01 M HCl for 2 h. Absorbance at 570 nm was measured using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA). Cell survival rates were expressed as a percentage of the ratio of A_{570} of cells treated with peptide to that of cells only.

Synergistic Effect. The synergistic effects between each peptide and antibiotic agents against *S. aureus* and MRSA were investigated by the combination assay. Two-fold serial dilutions of flavonoids were tested in the presence of a constant amount of peptide, equal to one-quarter and one-eighth of the MIC value of the peptides. Synergy was defined as occurring when the MIC of each of the drugs in the combination was one-quarter or less of the MIC of each drug alone.[34] To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination.[35] The following formula were used to calculate the FIC index: FIC of drug A = MIC of drug A in combination/MIC of drug A alone, FIC of drug B = MIC of drug B in combination/MIC of drug B alone, and FIC index = FIC of drug A + FIC of drug B. Synergy was defined as an FIC index ≤ 0.5. Indifference was defined as an FIC index of > 0.5 but of ≤ 4. Antagonism was defined as an FIC index of > 4.[34]

Results and Discussion

In order to design new potent peptides with antimicrobial and synergistic activities based on CAMA-P2, three analogues were synthesized as listed in Table 1. Parameters such as amphipathicity, net positive charge, α-helicity, and overall hydrophobicity have been shown to modulate the antibiotic activity of α-helical amphipathic antimicrobial peptides.[22,23,37] To investigate the effects of the total positive charges on antimicrobial activity, the $Ser^{14} \rightarrow$ Lys analogue (CAMA-syn1) was synthesized. The role of tryptophan at C-terminus on its antimicrobial activity was also investigated using $Ser^{14} \rightarrow Lys/Phe^{18} \rightarrow$ Trp analogue (CAMA-syn2). Also, we designed CAMA-syn3 by substitution of Lys^{16} located opposite side of substituted Lys^{14} of CAMA-syn1 with Leu residue, resulting in increase of hydrophobicity and amphipathicity of the peptide. The rank order of relative hydrophobicities of peptides was CAMA-syn2 < CAMA-syn1 < CAMA-P2 < CAMA < CAMA-syn3. CAMA-syn1 and CAMA-syn2 has higher net positive charge of +9.0 than the other peptides due to substitutions of the positively charged Lys residue for Ser residue.

We examined the antimicrobial activities of the peptides against a representative set of bacterial strains, including 3 gram-negative species (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) and 2 gram-positive species (*S. aureus* and *Bacillus subtilis*), and an antibiotic-resistant bacterial strains (MRSA (CCARM 3089)). The MIC values are shown in Table 2. All of the peptides showed effective MIC values similarly against all bacterial strain. The substitution of the Ser^{14} with Lys led to small increase of antimicrobial activities against Gram-positive bacteria. CAMA-syn3 with higher hydrophobicity has 2-fold increase of antimicrobial activity against MRSA.

We next assessed the hemolysis of the peptide against mammalian cells by measuring their abilities to cause lysis of human erythrocytes. Dose-response curves for the hemolytic activity of the peptides are shown in Figure 1. The CAMA-P2 peptide had the lowest hemolytic activity (1.7% at 100 μM) and CAMA and CAMA-syn1 showed a little higher hemolytic activities, 5.8% and 8.1% at 100 μM, respectively, compared with CAMA-P2, while CAMA-syn2 with $Ser^{14} \rightarrow Lys/Phe^{18} \rightarrow$ Trp substitution showed small increase of hemolytic activity, 20% at 100 μM. These peptides were not cytotoxic against red blood cell at their MIC values. However, CAMA-syn3 with $Ser^{14} \rightarrow Lys/Lys^{16} \rightarrow$ Leu substitution was hemolytic at its MIC.

The therapeutic potential of peptide antibiotic drugs lies in

Table 2. Antimicrobial activity of CAMA, CAMA-P2 and derivative peptides against standard bacterial strains and antibiotic-resistant bacterial strain

Peptide	MIC (μM)						Therapeutic index (MHC[a]/GM[b])
	E. coli	*P. aeruginosa*	*S. typhimurium*	*S. aureus*	*B. subtilis*	MRSA	
CAMA	2	1	2	4	4	4	17.7
CAMA-P2	2	1	2	4	4	4	35.3
CAMA-syn1	1	1	2	4	2	4	21.5
CAMA-syn2	2	1	2	2	2	4	11.5
CAMA-syn3	2	1	2	2	2	2	0.9
Melittin	2	1	4	4	4	4	0.06

[a]The minimal peptide concentration that produces hemolysis. [b]The geometric mean (GM) of the MIC values from all six bacterial strains in this table.

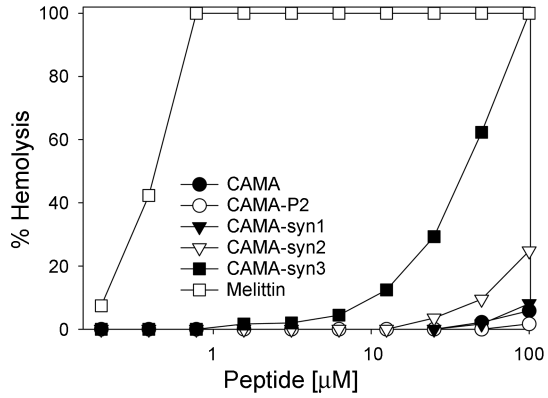


Figure 1. Dose-response of the hemolytic activity of the peptides toward human erythrocytes.

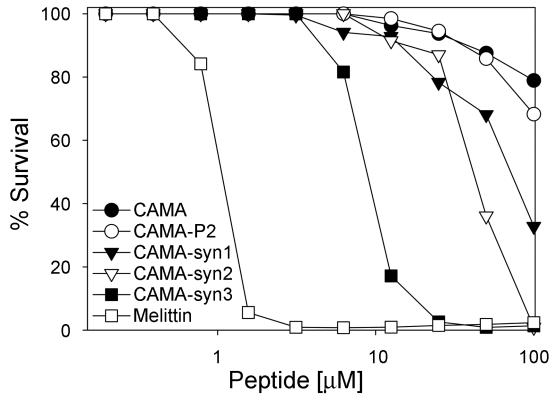


Figure 2. Growth inhibition dose-response curve for the peptides against Haca-T cells. Peptides are indicated as follows: CAMA (●), CAMA-P2 (○), CAMA-syn1 (▼), CAMA-syn2 (▽), CAMA-syn3 (■), and melittin (□).

the ability of the peptide to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. This property is conveyed by the concept of the therapeutic index, which is the ratio of the minimally effective concentration against h-RBCs to the minimally effective concentration against bacterial cells. These values are summarized in Table 2, which presents the minimal concentrations of peptides producing hemolysis of h-RBCs (MHCs), and the minimal concentrations that are toxic to bacteria (MICs), averaged across all six bacterial strains, to obtain the GM values that form the basis of the therapeutic index (MHC/GM). A high therapeutic index is thus an indication of two preferred characteristics of the peptide: a high MHC (low hemolysis) and a low MIC (high antimicrobial activity). All of CAMA-syn analogues had lower therapeutic index than CAMA-P2 while CAMA-syn1 had higher therapeutic index than CAMA. The CAMA-syn3 had the lowest therapeutic index among all peptides, implying that increase of hydrophobicity at C-terminal region caused the decrease in therapeutic index.

The cytotoxicities of the peptides against human keratinocyte Haca-T cells were measured, too. Effects on cell growth, which were assessed by measuring the mitochondrial conversion of MTT to a colored formazan product, are shown in Figure 2. CAMA-syn3 exhibited high cytotoxicity against Haca-T cells with IC_{50} value of 9.0 μM. Both CAMA-syn1 (IC_{50}, 75 μM) and CAMA-syn2 (IC_{50}, 45 μM) showed higher cytotoxicities than CAMA-P2 against Haca-T cells. CAMA as well as CAMA-P2 showed cell survival rate higher than

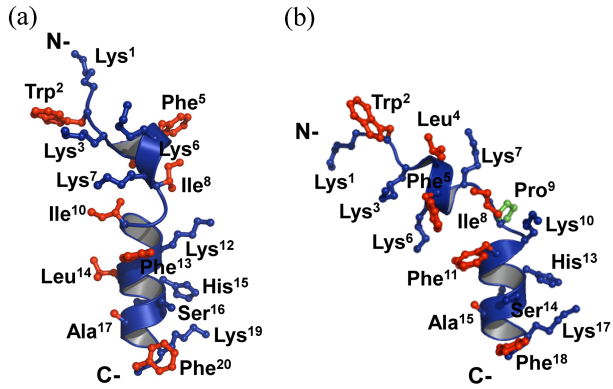


Figure 3. Ribbon diagram of CAMA (a) and CAMA-P2 (b). The hydrophobic and hydrophilic side-chains are in opposite directions with each other.

60% even at 100 μM.

We utilized our previous structures of CAMA (PDB entry 1F0D) and CAMA-P2 (PDB entry 1F0E) as determined by NMR spectroscopy to predict the structures of CAMA-syn1, CAMA-syn2, and CAMA-syn3.[22] Figure 3 shows the tertiary structures of CAMA and CAMA-P2. As shown in Figure 3(a), CAMA has a short amphiphilic helix in the N-terminus and about three turns of α-helix in the C-terminus, with the flexible hinge region in between in DPC micelle. In case of CAMA-P2, there are a short helix from Lys^3 to Lys^7 in the cecropin domain and an α-helix from Pro^9 to Phe^{18} in

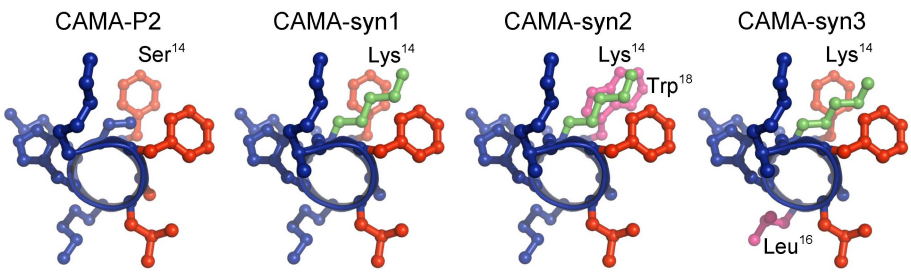


Figure 4. C-terminal end-on view of CAMA-P2, CAMA-syn1, CAMA-syn2 and CAMA-syn3. Structures of CAMA-syn1, CAMA-syn2 and CAMA-syn3 were predicted based on the structure of CAMA-P2. The hydrophobic side-chains are in right side and hydrophilic side-chains are in left side.

the magainin domain, connected with a flexible hinge region provided by Pro as shown in Figure 3(b). Figure 4 shows the orientation of the hydrophobic and hydrophilic side chains of the C-terminal helix of CAMA-P2 and its analogues (CAMA-syn1, CAMA-syn2 and CAMA-syn3). It is well known that when an amphipathic peptide forms an ion channel, the hydrophilic residues face inward to contact the solvent and the hydrophobic side chains face toward the acyl chains of the hydrophobic lipid. The hydrophobic side chains in these peptides, protrude toward right side, and the hydrophilic side chains protrude toward left side. In CAMA-syn1, Ser^{14} of CAMA-P2 was substituted with Lys, resulting in increase of cationicity. In CAMA-syn2, Phe^{18} located at C-terminus of CAMA-P2 was additionally substituted with Trp. Also, in CAMA-syn3, Lys^{16} located at the opposite side of the substituted Lys^{14} of CAMA-syn1 was substituted with Leu to increase hydrophobicity and amphipathicity. We have reported that the partial insertion of the Trp^2 of CAMA into the membrane, as well as the electrostatic interactions between the positively charged Lys residues at the N-terminus of the CAMA and the anionic phospholipid head groups, achieve the primary binding to the cell membrane. Then, the flexibility or bending potential induced by the Gly-Ile-Gly hinge sequence or the Pro residue in the central part of the peptides may allow the α-helix in the C-terminus to span the lipid bilayer. The analogues designed in this study may have the similar mechanism to that of CAMA. Furthermore, increase of cationicity facilitates CAMA analogues to interact with negatively charged phospholipid of bacterial cell membrane and Trp residue at C-terminus of CAMA-syn2 may be helpful to interact with phospholipid membrane. All of CAMA-syn analogues showed a little higher antimicrobial activities but also showed higher cytotoxicities against mammalian cells than parental peptide. It implies that optimum balance in cationicity, hydrophobicity, as well as amphipathicity is associated with antimicrobial activities and cytotoxcities of the peptides.

Recent reports have demonstrated that *in vitro* interaction of some cationic peptides with several clinically used antibiotics against several clinical isolates of Gram-positive and Gram-negative bacteria.[23-26] The use of combinations of antimicrobials is common in the clinical setting and expands the spectrum of organism that can be targeted, prevents the emergence of resistant organism, decreases toxicity by allowing lower doses of both agents and can result in synergistic inhibition.[27] We have previously screened potent antibiotic agents and demonstrated antimicrobial activities of naringenin, dihydrobinetin, and YKAs3001 as target of *Enterococcus faecalis* and *E. coli* KAS III, respectively.[38,39] We also found that cationic α-helical antimicrobial peptide, CAMA-syn, exhibited synergistic effects with YKAs3001 against *E. faecalis* and Vancomycin-resistant *Enterococcus faecalis* in the peptide corresponding to one-quarter of MIC value of peptide.[40] However, these experiments showed low antimicrobial activities against Gram-positive bacteria even though high binding affinity of these molecules with KAS III in fluorescence experiments.[38,39] The main reasons for these low antimicrobial activities are due to low membrane permeability of these polar antimicrobial agents.

We examined the antimicrobial activities of peptides to monitor whether they help antibiotic compounds to permeabilize membrane better as listed in Table 3. One-quarter and one-eighth of the MIC concentration of each peptide against *S. aureus* and MRSA was tested for combination assay. As shown in Table 3, all peptides showed a little improved MIC values with combination assay. We then investigated the synergistic effect by evaluating FIC index. It can be said that there is synergistic effect if an FIC index is less that 0.5.[41,42] Table 4 shows the values of FIC index for the combination of peptides and antimicrobial compounds. The two-dimen-

Table 3. Synergistic effect of the peptides with flavonoids against *S. aureus* and MRSA

Compound		MIC (μg/mL)					
		S. aureus			MRSA		
		YKAs 3001	Naringenin	Dihydrobinetin	YKAs 3001	Naringenin	Dihydrobinetin
Alone		64	64	1024	64	64	1024
CAMA	1/8[a]	64	64	512	64	64	1024
	1/4	32	64	128	32	64	256
CAMA-P2	1/8	64	64	1024	64	64	1024
	1/4	32	32	128	32	64	256
CAMA-syn1	1/8	64	64	1024	64	64	1024
	1/4	32	32	256	32	32	256
CAMA-syn2	1/8	32	64	1024	32	64	1024
	1/4	16	64	256	16	32	256
CAMA-syn3	1/8	64	64	512	64	64	1024
	1/4	64	64	128	32	64	256
Melittin	1/8	64	64	1024	64	64	1024
	1/4	64	64	128	64	64	256

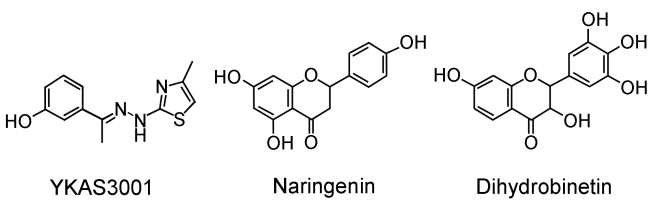
[a]All synergistic effects are determined in combination with amount of 1/8 or 1/4 of the peptide MIC.

Table 4. The values of FIC index for the combination of peptides and antimicrobial compound

Compound	FIC index[a]					
	S. aureus			MRSA		
	YKAs 3001	Naringenin	Dihydrobinetin	YKAs 3001	Naringenin	Dihydrobinetin
CAMA	1	2	0.375	1	2	0.5
CAMA-P2	1	1	0.375	1	2	0.5
CAMA-syn1	1	1	0.5	1	1	0.5
CAMA-syn2	0.5	2	0.5	0.5	1	0.5
CAMA-syn3	2	2	0.375	1	2	0.5
Melittin	2	2	0.375	2	2	0.5

[a]The FIC index was determined in the presence of a constant amount of peptide, equal to one-quarter of the peptide MIC.

sional structures of the antimicrobial compound for combination assay with peptides are depicted in Figure 5. To estimate the cell permeability of these antimicrobial compounds, we calculated topological polar surface area (TPSA) and *c*Log*P* using software of Molinspiration (http://www.molinspiration.com). *c*Log*P* is the partition coefficient between water and octanol as a factor of the lipophilicity of molecules.[43,44] PSA is defined as the surface sum over all polar atoms, in particular, TPSA is based on the summation of tabulated surface contributions of polar fragments.[45] These two properties are commonly used functions for determination of cell permeability in transport across the membranes.[45-47] As shown in Table 3 and 4, all of the peptides did not showed good synergistic effects with naringenin. In case of YKAs3001, only CAMA-syn2 showed synergistic effect (FIC index; 0.5) against all bacteria. Interestingly, all peptides exhibited synergistic effects with dihydrobinetin against both *S. aureus* and MRSA at one-quarter of MIC value of each peptide. *c*Log*P* of dihydrobinetin (0.50) is much smaller those of YKAs3001 (2.45) and naringenin (2.12). Therefore, the lipophilicity of dihydrobinetin is smaller than those of YKAs3001 and naringenin. As shown in Figure 5, dihydrobinetin has five hydroxyl groups and its TPSA (127.45 Å) is 2-fold or 1.5-fold larger than those of the YKAs3001 (57.51 Å) and naringenin (86.99 Å), respectively. The low lipophilicity and large TPSA are known as the common causes of poor cell permeability. Based on calculated *c*Log*P* and PSA, we can conclude that dihydrobinetin has difficulty to permeate the cell membrane compared with YKAs3001 and naringenin. CAMA, CAMA-P2, CAMA-syn1, and CAMA-syn3 showed synergistic effects (FIC index; 0.375) only with dihydrobinetin against *S. aureus* while CAMA-syn2 showed synergistic effects with YKAs3001 as well as dihydrobinetin. These results imply that substitution of Phe[18] with Trp in C-terminal helix of CAMA-syn2 may aid polar antimicrobial agents to permeate the bacterial membrane effectively.



YKAS3001 Naringenin Dihydrobinetin

Figure 5. The chemical structures of YKAs3001, naringenin and dihydrobinetin used in combination assay with antimicrobial peptides.

Conclusion

In this study, we tried to develop peptide antibiotics with increased antimicrobial and synergistic activity with low cytoxicity by designing hybrid peptide analogues based on structure-activity relationships of CAMA peptides. The CAMA-syn1 with substitution of Ser[14] with Lys in CAMA-P2 retaining the structure of CAMA-P2 with increase of cationicity at C-terminal region showed antimicrobial and hemolytic activity similar with CAMA-P2 while this peptide showed higher cytotoxicity against Haca-T cells than CAMA-P2, but it did not showed any toxicity at its MIC. Analogue, CAMA-syn3 with Ser[14] → Lys/Lys[16] → Leu substitution has 2-fold increase of antimicrobial activity against MRSA by increasing hydrophobicity in C-terminal helix, but it showed higher hemolytic activity and cytotoxicity against Haca-T cells compared to other peptides, implying that increase of hydrophobicity at C-terminal region caused increase in cytoxicity of peptide analogues. Also, to investigate the effects of Trp residue at C-terminal helix on antimicrobial and synergistic activity of CAMA-P2 analogues, we synthesized CAMA-syn2 with Ser[14] → Lys/Phe[18] → Trp substitution. CAMA-syn2 showed similar antimicrobial activity to that of CAMA-syn1 and small increase of hemolytic activity compared to CAMA-P2 peptide. All peptides showed a little increase of antimicrobial activities compared to their parent peptides, CAMA and CAMA-P2 as well as melittin.

In combinations assay of peptides with antibiotic compounds, YKAs3001 and antimicrobial flavonoids such as naringenin and dihydrobinetin, all of the peptides exhibited synergistic effects with dihydrobinetin having low lipophilicity and large TPSA against both *S. aureus* and MRSA. Particularly, only CAMA-syn2 exhibited synergistic effects with YKAs3001 against bacteria suggesting that Trp residue at C-terminus of CAMA-syn2 may be important factor for improving membrane permeabilization of the compounds,

resulting in increase of synergistic effect. This study will be useful for our efforts to design novel antimicrobial peptides with potent antibiotic activity and synergistic activity without cytotoxicity.

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