Effect of Double Replacement of L-Pro, D-Pro, D-Leu or Nleu in Hydrophobic Face of Amphipathic α-Helical Model Antimicrobial Peptide on Structure, Cell Selectivity and Mechanism of Action

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In order to investigate the effects of the double replacement of L-Pro, D-Pro, D-Leu or Nleu (the peptoid residue for Leu) in the hydrophobic face (positions 9 and 13) of amphipathic α-helical non-cell-selective antimicrobial peptide L8K9W1 on the structure, cell selectivity and mechanism of action, we synthesized a series of L8K9W1 analogs with double replacement of L-Pro, D-Pro, D-Leu or Nleu in the hydrophobic face of L8K9W1. In this study, we have confirmed that the double replacement of L-Pro, D-Pro, or Nleu in the hydrophobic face of L8K9W1 led to a great increase in the selectivity toward bacterial cells and a complete destruction of α-helical structure. Interestingly, L8K9W1-D-Pro, L8K9W1-D-Pro and L8K9W1-Nleu preferentially interacted with negatively charged phospholipids, but unlike L8K9W1 and L8K9W1-D-Leu, they did not disrupt the integrity of lipid bilayers and depolarize the bacterial cytoplasmic membrane. These results suggested that the mode of action of L8K9W1-L-Pro, L8K9W1-D-Pro and L8K9W1-Nleu involves the intracellular target other than the bacterial membrane. In particular, L8K9W1-L-Pro, L8K9W1-D-Pro and L8K9W1-Nleu had powerful antimicrobial activity (MIC range, 1 to 4 μM) against methicillin-resistant Staphylococcus aureus (MRSA) and multidrug-resistant Pseudomonas aeruginosa (MDRPA). Taken together, our results suggested that L8K9W1-L-Pro, L8K9W1-D-Pro and L8K9W1-Nleu with great cell selectivity may be promising candidates for novel therapeutic agents, complementing conventional antibiotic therapies to combat pathogenic microorganisms.

Key Words : Model antimicrobial peptide, Structure, Cell selectivity, Bacterial-killing mechanism

Introduction

Antimicrobial peptides (AMPs) are non-adaptive host defense components that provide a first line of defense against invading pathogens.1-3 AMPs are widely distributed in all species ranging from protozoa to vertebrates.1,2,4 With the alarming emergence of a number of bacteria that are resistant to conventional antibiotics, antimicrobial peptides are being intensively studied with the objective of developing a novel class of antimicrobial drugs.1-3 AMPs are classified into cell-selective and non-cell-selective peptides. The cell-selective peptides (e.g. magainins and cecropins) selectively kill bacterial cells but not mammalian cells.5,6 The non-cell-selective (e.g. melittin and pardaxin) kill both bacterial and mammalian cells.6,7 The cell selectivity between AMPs is due to the difference in the interaction between zwitterionic (major component of mammalian cells) and negatively charged (major component of bacterial cells) phospholipids. The non-cell-selective AMPs bind both zwitterionic and negatively charged phospholipid membranes, whereas the cell-selective peptides preferentially bind negatively charged phospholipids. For AMPs to be useful in human and veterinary application, these peptides must have high selectivity toward bacterial cells.

Even though the exact mechanism against target microorganisms of AMPs is not clearly understood, it is generally accepted that it involves interaction of the peptides with the lipid membranes of the target microbe.8,9 First the positively charged residues of the peptides bound to the negative charged phospholipid of the pathogen mainly the electrostatic interactions. And then, they mostly adapted α-helical structure or β-sheet structure and increased the permeability of the lipid membranes either by ion channel formation or the disruption of lipid bilayers, resulting in the cell death of target cells. In addition, the hydrophobic interactions between hydrophobic residues of the peptide and the lipid acyl chain are essential for their ion channel formation or the disruption of lipid bilayers. Accordingly, amphipathic α-helical LK-model peptides composed of positively charged lysines and hydrophobic leucines have been employed to clarify their modes of action and selectivity between bacterial and mammalian cells.10-19 Many studies using α-helical LK-model peptides indicated that net positively charge and hydrophobicity of the peptides is sufficient for showing antimicrobial activity and their hydrophobic-hydrophilic balance is a critical factor in controlling the selectivity between bacterial and mammalian cells.10-19

In our previous study, in order to develop novel cell-selective peptides having potent antimicrobial activity but no hemolytic activity and investigate their mode of action on bacterial and mammalian cells, we designed and synthesized a perfectly amphipathic α-helical non-cell selective LK-
the helix-breaking amino acid such as composed of hydrophobic residues (Leu and Trp) and more, the molecular mechanism of bacterial-killing action of bacterial and mammalian cytoplasmic membranes. Further-of the peptides with model liposome systems mimicking the bacterial and mammalian cells, we examined the interaction cular basis for the selectivity of the peptides between the membrane-mimicking environments. To investigate the molecular mass (Da)

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino acid sequences</th>
<th>Calculated</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(_8)K(_9)W(_1)</td>
<td>KWKLLKLLKLLKLLKLLK-NH(_2)</td>
<td>2262.1</td>
<td>2262.3</td>
</tr>
<tr>
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<td>2262.3</td>
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<td>KWKLLKLLKPLKLLKLLK-NH(_2)</td>
<td>2262.1</td>
<td>2261.9</td>
</tr>
</tbody>
</table>

P: t-Pro, \(\mathbf{P}:\) d-Pro, \(\mathbf{L}:\) d-Leu, \(I:\) Nleu [(CH\(_3\))\(_2\)-CH-NH-CH\(_2\)-COOH] (Peptoid residue for Leu)
(18.5:5:5:2.5:1, v/v/v/v/v/v) for 2 h at room temperature, the crude peptide was repeatedly extracted with diethyl ether and purified by RP-HPLC (reverse-phase high-performance liquid chromatography) on a preparative Vydac C18 column (20 mm × 250 mm, 300 Å, 15-μm particle size) using an appropriate 0–90% water/acetoniitrite gradient in the presence of 0.05% trifluoroacetic acid. The final purity of the peptides (> 95%) was assessed by RP-HPLC on an analytical Vydac C18 column (4.6 × 250 mm, 300 Å, 5-μm particle size). Peptides had the correct atomic mass, as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Table 1).

Antimicrobial Assay. The antimicrobial activity of the peptides against three Gram-positive bacterial strains, three Gram-negative bacterial strains and three MRSA strains was examined by using the broth microdilution method in sterile 96-well plates. Aliquots (100 μL) of a bacterial suspension at 2 × 10^6 colony-forming units (CFU)/mL in 1% peptone were added to 100 μL of the peptide solution (serial 2-fold dilutions in 1% peptone). After incubation for 18-20 h at 37 °C, bacterial growth inhibition was determined by measuring the absorbance at 600 nm with a Microplate Auto-reader EL 800 (Bio-Tek Instruments, VT). The minimal inhibitory concentration (MIC) was defined as the minimum peptide concentration that inhibited bacteria growth. Three types of Gram-positive bacteria (Bacillus subtilis [KCTC 3068], Staphylococcus epidermidis [KCTC 1917] and Staphylococcus aureus [KCTC 1621]) and three types of Gram-negative bacteria (Escherichia coli [KCTC 1682], Pseudomonas aeruginosa [KCTC 1637] and Salmonella typhimurium [KCTC 1926]) were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRBIB). Methicillin-resistant Staphylococcus aureus (MRSA) (CCARM 3001 and CCARM 3543) and multidrug-resistant Pseudomonas aeruginosa (MDRPA) (CCARM 2095) were obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women’s University in Korea.

Quantification of Hemolytic Activity. Fresh human red blood cells (hRBCs) were centrifuged, washed three times with phosphate-buffered saline (PBS) (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4), dispensed into 96-well plates as 100 μL of 4% (w/v) hRBC in PBS, and 100 μL of peptide solution was added to each well. Plates were incubated for 1 h at 37 °C, then centrifuged at 1000 × g for 5 min. Samples (100 μL) of supernatant were transferred to 96-well plates and hemoglobin release was monitored by measuring absorbance at 414 nm. Zero hemolysis was determined in PBS (AsPBS) and 100% hemolysis was determined in 0.1% (v/v) Triton X-100 (Atriton). The hemolysis percentage was calculated as: % hemolysis = 100 × [(Asample – AsPBS)/(Atriton – AsPBS)].

Circular Dichroism (CD) Spectroscopy. The CD spectrum of the peptide was obtained with a Jasco J-715 CD spectrophotometer (Tokyo, Japan) at 25 °C using a fused quartz cell with a 1-mm path length over a wavelength range of 190–250 nm at 0.1 nm intervals (speed, 50 nm/min; response time, 0.5 s; bandwidth, 1 nm). CD spectra were collected and averaged over four scans. Samples were prepared by dissolving the peptide to a final concentration of 100 μg/mL in 10 mM sodium phosphate buffer (pH 7.2), 50% (v/v) trifluoroethanol (TFE) or 30 mM sodium dodecyl sulfate (SDS). The mean residue ellipticity [θ] (measured as units of deg cm^2 dmol^-1) was calculated using the formula: [θ] = [θ]_obs (MRW/10lε), where [θ]_obs is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in mg/mL, and l is the optical path length of the cell in cm. Spectra were expressed as molar ellipticity [θ] versus wavelength.

Tryptophan Fluorescence and Quenching. Small unilamellar vesicles (SUWs) were prepared for tryptophan fluorescence experiments as described previously.21 Following chloroform evaporation, the EYPE/EYPG (7:3, w/w) or EYPC/cholesterol (10:1, w/w) lipids were resuspended in 10 mM Tris–HCl buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA) by vortexing. The lipid dispersions were sonicated in ice water for 20 min using an ultrasonic cleaner until the solutions clarified. Tryptophan fluorescence spectra were measured using an RF 5301 fluorescence spectrophotometer (Shimadzu, Japan). The procedure was performed for each peptide in 10 mM Tris–HCl buffer (pH 7.4) with 500 μM EYPE/EYPG or EYPC/cholesterol lipids. The peptide/lipid molar ratio was 1:50, and the peptide/liposome mixture was allowed to interact for 2 min at 25 °C. An excitation wavelength of 280 nm was used, and emission was scanned at wavelengths ranging from 300 to 400 nm. The spectra were baseline corrected by subtracting blank spectra of the corresponding solutions without the peptide. The quenching of fluorescence was accomplished using acrylamide. To reduce the absorbance of acrylamide, Trp was excited at 295 nm instead of 280 nm. The final concentration of acrylamide was brought to 0.21 M by titrating the 4 M stock solution with liposomes at a lipid/peptide molar ratio of 50:1. The effects of the quenching reagent on peptide fluorescence intensities were assessed by the quenching constant (KSV), which was estimated using the Stern–Volmer equation: \( F_0/F = K_{sv}Q \), where \( F_0 \) and \( F \) are the fluorescence values of the peptide in the absence or the presence of acrylamide, respectively, \( K_{sv} \) represents the Stern–Volmer quenching constant, and \( Q \) represents the concentration of acrylamide.

Dye Leakage. Calcein-entrapped large unilamellar vesicles (LUVs) composed of EYPE/EYPG (7:3, w/w) were prepared by vortexing the dried lipid in a dye buffer solution (70 mM calcin, 10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was subjected to 10 freeze-thaw cycles in liquid nitrogen and extruded 21 times through polycarbonate filters (2 stacked 100-nm pore size filters) with a LiposoFast extruder (Avestin, Inc. Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. Calcein leakage from LUVs was monitored by measuring the fluorescence intensity at an excitation wavelength of 490 nm and emission wavelength at 520 nm on a model RF-5301PC spectrophotometer. Complete dye release
was obtained using 0.1% Triton X-100.

Membrane Depolarization. The cytoplasmic membrane depolarization activity of the peptides was measured using the membrane potential sensitive dye, diSC3-5 as previously described. Briefly, *Staphylococcus aureus* (KCTC 1621) grown at 37 °C with agitation to the mid-log phase (OD600 = 0.4) was harvested by centrifugation. Cells were washed twice with washing buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended to an OD600 of 0.05 in similar buffer. The cell suspension was incubated with 20 nM diSC3-5 until stable reduction of fluorescence was achieved, implying incorporation of the dye into the bacterial membrane. Then KCl was added to a final concentration of 0.1 M to equilibrate K+ levels. Membrane depolarization was monitored by recording changes in the intensity of fluorescence emission of the membrane potential-sensitive dye, diSC3-5 (excitation λ = 622 nm, emission λ = 670 nm) after peptide addition. The membrane potential was fully dissipated by adding gramicidin D (final concentration of 0.2 nM). The membrane potential dissipating activity of the peptides was calculated as follows:

% Membrane depolarization = 100 × [(F₀ - F₆) / (F₆ - F₀)]

where F₀ denotes the stable fluorescence value after the addition of the diSC3-5 dye, F₆ denotes the fluorescence value 5 min after peptide addition, and F₆ denotes the fluorescence signal after gramicidin D (GD) addition.

Results and Discussion

Peptide Synthesis. The structure and molecular weight of the peptides were verified by MALDI-TOF MS. Table 1 summarizes the theoretically calculated and measured molecular weight of each peptide. All peptides had molecular weight values in agreement with their theoretical values, suggesting that the peptides were successfully synthesized.

Antimicrobial Activity. The antimicrobial activities of the peptides were tested in terms of their bacteriostatic activity (L₉₀) and multidrug-resistant *Staphylococcus aureus* (MDRPA). As shown in Table 3, L₉₀₁-W₁-Pro, L₉₀₁-W₁-D-Pro and L₉₀₁-W₁-Nleu (MIC range, 1 to 4 µM) were more active to MRSA and MDRPA than L₉₀₁-W₁ and L₉₀₁-W₁-D-Leu (MIC range, 4 to 8 µM).

Cell Selectivity. Therapeutic index (TI) is a widely employed parameter to represent the cell selectivity of antimicrobial reagents. It was calculated by the ratio of MHC (the peptide concentration that causes 10% hemolysis) and GM (geometric mean of MICs against six bacterial strains) tested compared to L₉₀₁-W₁. The potency of antimicrobial activity of the peptides was ranked according to the geometric mean (GM) of the MICs as follows: L₉₀₁-W₁-Nleu > L₉₀₁-W₁-D-Pro > L₉₀₁-W₁-L-Pro > L₉₀₁-W₁-D-Leu > L₉₀₁-W₁.

Hemolytic Activity. The cytotoxicity of the peptides is often analyzed in terms of their ability to lyse mammalian red blood cells, referred to as hemolysis. The ability of the peptides tested in this study to induce hemolysis of human erythrocytes is summarized in Table 2. Both L₉₀₁-W₁ and L₉₀₁-W₁-D-Leu were found to be relatively highly hemolytic activity (L₉₀₁-W₁ and L₉₀₁-W₁-D-Leu induced 10% hemolysis at 1.36 µM and 1.38 µM, respectively). However, L₉₀₁-W₁-L-Pro, L₉₀₁-W₁-D-Pro and L₉₀₁-W₁-Nleu had no hemolytic activity at concentrations as high as 100 µM.

Antimicrobial Activity Against Antibiotic-resistant Bacteria. We tested the antimicrobial activity of the peptides against methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA). As shown in Table 3, L₉₀₁-W₁-L-Pro, L₉₀₁-W₁-D-Pro and L₉₀₁-W₁-Nleu (MIC range, 1 to 4 µM) were more active to MRSA and MDRPA than L₉₀₁-W₁ and L₉₀₁-W₁-D-Leu (MIC range, 4 to 8 µM).

Table 3. Antimicrobial activities of the peptides against antibiotic-resistant bacteria

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Minimal Inhibitory Concentration (MIC) (µM)</th>
</tr>
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<tr>
<td></td>
<td>MRSA (CCARM 3001)</td>
</tr>
<tr>
<td>L₉₀₁-W₁</td>
<td>4</td>
</tr>
<tr>
<td>L₉₀₁-W₁-L-Pro</td>
<td>4</td>
</tr>
<tr>
<td>L₉₀₁-W₁-D-Pro</td>
<td>2</td>
</tr>
<tr>
<td>L₉₀₁-W₁-D-Leu</td>
<td>4</td>
</tr>
<tr>
<td>L₉₀₁-W₁-Nleu</td>
<td>2</td>
</tr>
</tbody>
</table>

*Antibiotic-resistant bacterial strains were clinically isolated at the Culture Collection of Antibiotic-Resistant Microbes (CCARM). MRSA: Methicillin-resistant *Staphylococcus aureus*. MDRPA: multidrug-resistant *Pseudomonas aeruginosa*.

Table 2. Minimum inhibitory concentrations (MIC), minimum hemolytic concentrations (MHC), and therapeutic indices (TI) of the peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Minimal Inhibitory Concentration (MIC) (µM)</th>
<th>Gram-negative bacteria</th>
<th>Gram-positive bacteria</th>
<th>GM (µM)</th>
<th>MHC (µM)</th>
<th>TI (MHC/GM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L₉₀₁-W₁</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1.36</td>
</tr>
<tr>
<td>L₉₀₁-W₁-L-Pro</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1.33</td>
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<tr>
<td>L₉₀₁-W₁-D-Pro</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>1.25</td>
</tr>
<tr>
<td>L₉₀₁-W₁-D-Leu</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2.33</td>
</tr>
<tr>
<td>L₉₀₁-W₁-Nleu</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*MICs were determined as the lowest concentration of peptide that causes 100% inhibition of microbial growth. GM is the geometric mean (GM) of the peptide MICs against all six bacterial strains. MHC is the minimum hemolytic concentration that caused 10% hemolysis of human red blood cells (hRBC). When no detectable hemolytic activity was observed at 100 µM, a value of 200 µM was used to calculate the therapeutic index. Therapeutic index (TI) is the ratio of the MHC to the geometric mean of MIC (GM). Larger values indicate greater cell selectivity.
strains). When there was significant no hemolysis at the highest concentration tested (100 μM), 200 μM was used for the TI calculation, since the test was carried out by twofold serial dilution. Larger values in TI indicate greater cell selectivity. As shown in Table 2, L8K9W1 and L8K9W1-D-Leu had much lower TI of 0.34 and 0.38, respectively, providing poor cell selectivity. In contrast, L8K9W1-L-Pro, L8K9W1-D-Pro and L8K9W1-Nleu exhibited a great TI of 150.4 and 160.0, offering great cell selectivity.

Secondary Structure. Circular dichroism (CD) was performed for all four peptides in different media, including aqueous solutions and membrane-like environments (50% TFE and 30 mM SDS) (Figure 2). In sodium phosphate buffer, all peptides displayed spectra typical of an unordered conformation. In the presence of 50% TFE and 30 mM SDS, L8K9W1 and L8K9W1-D-Leu displayed the typical α-helical structure characterized by the positive bands near 195 nm and the dichroic minimal values at 205–208 and 222–223 nm.
nm. In contrast, L9K8W7-l-Pro, L9K8W7-d-Pro and L9K8W7-Nleu had an unordered conformation. This result indicated that the double replacement of l-Pro, d-Pro, or Nleu in the hydrophobic face of amphipathic α-helical L9K8W7 let to a complete destruction of α-helical structure.

**Tryptophan Fluorescence and Quenching.** The fluorescence emission characteristics of the Trp residues in the peptides are sensitive to their environment and were used to monitor the binding of peptides to lipid vesicles. The liposomal EYPE/EYPG (7:3, w/w) and EYPC/cholesterol (10:1, w/w) vesicles generally mimic bacterial and eukaryotic membranes, respectively. Fluorescence emission spectra for the tryptophan of the peptides in the presence of phospholipid vesicles are shown in Figure 3. All peptides caused maximal fluorescence emission at approximately 350 nm in aqueous solutions (Table 4). L9K8W7 and L9K8W7-d-Leu induced larger blue shifts in both EYPE/EYPG (7:3, w/w) and EYPC/cholesterol (10:1, w/w) vesicles, which was consistent with its high antimicrobial activity and high hemolytic activity (Table 4). In contrast, L9K8W7-l-Pro, L9K8W7-d-Pro and L9K8W7-Nleu induced larger blue shifts in EYPE/EYPG (7:3, w/w) but no blue shift in EYPC/cholesterol (10:1, w/w) vesicles, correlating with their high antimicrobial activity and no hemolytic activity (Table 4). The location of the peptides with respect to the bilayer plane was further investigated using fluorescence quenching techniques to create Stern–Volmer plots of the decrease in fluorescence as a function of an added soluble quencher (Figure 4 and Table 4). L9K8W7-l-Pro, L9K8W7-d-Pro and L9K8W7-Nleu had the same low $K_{SV}$ (1.77 and 1.99) in negatively charged vesicles, but much higher $K_{SV}$ (3.76-4.14) in zwitterionic vesicles as compared to L9K8W7 and L9K8W7-d-Leu, which was in agreement with their greatest cell selectivity (Table 4).

**Fluorescent Dye Leakage from Bacterial Membrane Mimicking Liposome.** To determine whether the antimicrobial activities of the peptides depend on their capacity to permeate bacterial membranes, we measured their abilities to induce the fluorescent dye calcein leakage from negatively charged EYPE/EYPG (7:3, w/w) large unilamellar vesicles (LUVs), which mimics bacterial membranes. As shown in Figure 5, L9K8W7 and L9K8W7-d-Leu induced a significant dye leakage (more than 50%) at 1.0 μM. In contrast, L9K8W7-l-Pro, L9K8W7-d-Pro and L9K8W7-Nleu had very little leakage (less than 12%) at concentrations as high as 12.0 μM. This result suggested that bacterial-killing mechanism of L9K8W7-l-Pro, L9K8W7-d-Pro and L9K8W7-Nleu is not due to the disruption/perturbation of bacterial cytoplasmic membranes.

**Membrane Depolarization.** The membrane potential sensitive dye diSC3-5 was used to monitor the cytoplasmic membrane depolarization of *Staphylococcus aureus* cells in the presence of peptides. This dye is distributed between the cells and medium, depending on the cytoplasmic membrane

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**Table 4.** Fluorescence spectroscopy parameters measured for the peptides in the presence and absence of EYPE/EYPG (7:3, w/w) and EYPC/cholesterol (10:1, w/w) vesicles

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fluorescence emission maxima (nm)$^a$</th>
<th>$K_{SV}^b$ (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
<td>EYPE/EYPG</td>
</tr>
<tr>
<td>L9K8W7-l-Pro</td>
<td>344</td>
<td>331 (13)</td>
</tr>
<tr>
<td>L9K8W7-d-Pro</td>
<td>350</td>
<td>335 (15)</td>
</tr>
<tr>
<td>L9K8W7-d-Leu</td>
<td>350</td>
<td>336 (14)</td>
</tr>
<tr>
<td>L9K8W7-Nleu</td>
<td>350</td>
<td>331 (19)</td>
</tr>
</tbody>
</table>

$^a$Blue shift of emission maximum as compared to Tris-buffer. $^b$Stern–Volmer constant $K_{SV}$ was calculated by the Stern–Volmer equation: $F_0/F = 1/K_{SV} (Q)$, where $Q$ is the concentration of the quencher (acrylamide). Concentrations of the quencher were increased from 0.03 to 0.21 M. A smaller $K_{SV}$ value reflects a more protected Trp residue.
Figure 5. Peptide-induced calcein release from calcein-entrapped negatively charged EYPE/EYPG (7:3, w/w) LUVs. Symbols: LaKoW1 (●), LaKoW1-D-Pro (○), LaKoW1-D-Pro (▼), LaKoW1-D-Leu (▲) and LaKoW1-Nleu (■).

Figure 6. Concentration-dependent, peptide-induced cytoplasmic membrane depolarization against Staphylococcus aureus. Membrane depolarization was measured by an increase in fluorescence of the potentiometric fluorescent dye dSC6-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Symbols: LaKoW1 (●), LaKoW1-D-Pro (○), LaKoW1-D-Pro (▼), LaKoW1-D-Leu (▲) and LaKoW1-Nleu (■).

Figure 7. Time-dependent, peptide-induced cytoplasmic membrane depolarization against Staphylococcus aureus. Membrane depolarization was measured by an increase in fluorescence of the membrane potential–sensitive dye, dSC6-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. In each run, the peptides were added near the 4.4-min mark.

released into the medium, causing a measurable increase in fluorescence. Both LaKoW1 and LaKoW1-D-Leu induced a complete membrane depolarization at 0.2 μM (Figures 6 and 7). In contrast, LaKoW1-D-Pro, LaKoW1-D-Pro and LaKoW1-Nleu caused no or less membrane depolarization even at 2 μM (Figures 6 and 7). This result suggested that LaKoW1-D-Pro, LaKoW1-D-Pro and LaKoW1-Nleu did not kill microorganisms by the formation of pore/ion channels on bacterial cell membranes.

Conclusion

In this study, we have confirmed that the double replacement of L-Pro, D-Pro or Nleu in the hydrophobic face (positions 9 and 13) of non-cell-selective AMP LaKoW1 let to a great increase in the selectivity toward bacterial cells and a complete destruction of α-helical structure. Interestingly, LaKoW1-D-Pro, LaKoW1-D-Pro and LaKoW1-Nleu do not disrupt membranes to a large enough extent to allow for leakage of calcein from bacterial membrane-mimicking LUVs or extensive membrane depolarization. These results suggested that the mode of action of LaKoW1-D-Pro, LaKoW1-D-Pro and LaKoW1-Nleu involves the intracellular target other than the bacterial membrane. In particular, LaKoW1-D-Pro, LaKoW1-D-Pro and LaKoW1-Nleu displayed strong antimicrobial activity (MIC range, 1 to 4 μM) against MRSA and MDRPA. Taken together, our results suggested that LaKoW1-D-Pro, LaKoW1-D-Pro and LaKoW1-Nleu with great cell selectivity appear to be promising candidates for future development as novel antimicrobial agents.

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References

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