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February 2012

Ph.D. Dissertation

Cell Selectivity, Mechanism of Action and Anti-inflammatory Activity of Cathelicidin-derived and Model Antimicrobial Peptides

Graduate School of Chosun University

Department of Bio-Materials

Yonghai Nan

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카텔리시딘 유래 및 모델 항균펩타이드의 세포선택성, 작용기작 및 항염증활성

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Cell Selectivity, Mechanism of Action and Anti-inflammatory Activity of Cathelicidin-derived and Model Antimicrobial Peptides

Advisor: Prof. Song Yub Shin

This dissertation is submitted to the Graduate School of Chosun Univeristy in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Science

October 2011

Graduate School of Chosun University

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Yonghai Nan's Ph.D Dissertation has certified.

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PART Ⅰ

인간의 cathelicidin 항균펩타이드(AMP), LL-37은 lipopolysaccharide (LPS)에 의해 유도된 cellular cytokine의 생성을 억제하고 또한 항균작용을 나타낸다. LL-37과 비교했을 때 보다 짧은 서열을 가지며 prokaryotic selectivity을 증가시키고, 항-염증활성을 유지하는 새로운 항균 펩타이드를 개발하기 위해 IG-19 (LL-37의 13-31잔기)를 기초로 하여 아미노산을 치환한 유사체들을 합성하였다. IG-19 과 그것의 유사체들의 prokaryotic selectivity [therapeutic index (TI)로 나타냄)]와 항-염증활성은 펩타이드의 hydrophobicity와 중요한 직접적인 연관이 있었다. IG-19의 유사체들 중에서 a4는 제일 높은 prokaryotic selectivity (a4는 of LL-37보다 7.1배 높은 therapeutic index를 나타냈었다)를 보여주었지만 LL-37과 비교했을 때 낮은 항-염증활성을 나타냈다. 높은 hydrophobicity를 가진 a5, a6, a7, a8과 a9 유사체들은 LL-37과 비교했을 때 강한 항-염증활성을 가졌지만 약한 prokaryotic selectivity (TI: 0.1~0.5)를

나타냈다. 이러한 결과는 펩타이드의 적당한 hydrophobicity가 prokaryotic selectivity와 항-염증활성에 매우 중요하다는 것을 제시한다. 그리고 a4 유사체의 prokaryotic selectivity를 유지하면서 항-염증활성을 증가시키기 위해, a4 유사체의 Phe^{15} 또는 Phe^{5}를 각각 Trp으로 치환한 유사체 (a4-W1과 a4-W2)를 합성하였다. a4-W1과 a4-W2는 net positive charge +11과 비슷한 hydrophobicity와 α-helicity를 가지고 있음에도 불구하고 이들 두 펩타이드는 동일한 prokaryotic selectivity를 나타내지만 a4-W1는 a4-W2와 비교했을 때 더욱 높은 항-염증활성을 나타냈다. 이러한 결과는 prokaryotic selectivity를 크게 감소시키지 않고, 높은 항-염증 활성을 가진 prokaryotic-selective 항균 펩타이드를 설계할 때 가장 유효한 Trp의 치환위치는 펩타이드의 α-helical wheel projection에서 친수성 끝나는 면과 소수성 시작면 사이의 양쪽 친매성의 경계면이라는 것을 제시하였으며, 또한 항균 펩타이드의 항-염증활성에는 net positive charge와 hydrophobicity 뿐만 아니라 다른 중요한 parameter들이 존재한다는 것을 제시한다. 그 외에도 펩타이드의 가수분해효소 절단 (proteolytic digestion)에 대한 안정성을 제공하기 위하여 a4-W1과 a4-W2의 3, 7, 10, 13 및 17의 위치에 D-아미노산을 치환한 diastereomeric 펩타이드(a4-W1-D와 a4-W2-D) 및 D-enantiomeric 펩타이드 (a4-W1-E와 a4-W2-E)를 합성하였다. Tryptic digestion후에도 diastereomeric 및 D-enantiomeric 펩타이드는 그들의 항균활성을 유지하였다. D-diastereomeric 펩타이드는 높은 prokaryotic selectivity와 protease resistance을 보여주었지만 낮은 항-염증반응을 나타냈다. D-enantiomeric펩타이드는 prokaryotic selectivity, 항-염증활성 및 protease resistance를 모두 나타내었다.

결과적으로 a4-W1, a4-W1-E와 a4-W2-E는 LL-37과 비교했을 때, 증가된 prokaryotic selectivity와 유지된 항-염증활성을 나타내었으므로 이들 펩타이드들은 미생물 감염뿐 만 아니라 폐혈증 치료를 위한 유용한 항균제로서의 발전 가능성이 있음을 제시한다.

PART Ⅱ

α-helical homo-dimeric항균 펩타이드의 disulphide bond의 위치가 염-저항성(salt resistance)과 LPS의 중성화에 미치는 영향을 조사하기 위하여, α-helical 모델 펩타이드인 $K_6L_4W_1$ 와 그것의 disulphide bond가 각각 아미노말단, 서열중앙위치, 카르복실말단에 있는 homo-dimeric 펩타이드인 di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M와 di-$K_6L_4W_1$-C를 합성하였다. $K_6L_4W_1$ 및 di-$K_6L_4W_1$-M과 다르게 $K_6L_4W_1$-N 및 di-$K_6L_4W_1$-C의 항균활성은 150 mM NaCl에서 영향을 받지 않았다. di-$K_6L_4W_1$-M과 비교했을 때 di-$K_6L_4W_1$-N 및 di-$K_6L_4W_1$-C는 LPS로 자극한 쥐의 대식세포인 RAW264.7 세포에서 일산화질소(nitric oxide)의 생성을 아주 크게 억제하였다. 결론적으로 펩타이드 분자의 disulphide bond가 아미노말단 또는 카르복실말단에 위치하는 것이 서열중앙위치에 위치하는 것 보다 항균활성과 LPS중성화 능력을 가진 염-저항성 α-helical homo-dimeric 항균 펩타이드를 설계하는데 더욱 효과적이라는 것을 제시한다.

PART Ⅲ

Arg 또는 Lys을 함유하고 Trp이 풍부한 모델 항균 펩타이드 ($K_6L_2W_3$ 및 $R_6L_2W_3$)와 그들의 D-enantiomeric 펩타이드 ($K_6L_2W_3$-D 및 $R_6L_2W_3$-D)에 대하여 mammalian 세포독성과 항균활성의 기작에 대하여 조사하였다. Arg을 함유한 펩타이드는 Lys이 함유된 펩타이드와 비교했을 때 인간 적혈구(human erythrocytes)와 mammalian cells에서 더욱 강한 독성을 가졌다. 역상 액체크로마토그래피 (RP-HPLC)의 지연시간(retention time)에서 판단하였을 때, Arg이 함유한 펩타이드는 Lys이 함유한 펩타이드 보다 더욱 강한 hydrophobicity를 나타내었다. 이러한 결과는 펩타이드의 약간의 hydrophobicity 의 차이는 그들의 용혈활성과 mammalian 세포독성에 영향 준다는 것을 말해준다. 흥미롭게도 $K_6L_2W_3$와 $K_6L_2W_3$-D는 거의 비슷한 mammalian 세포독성을 나타내지만, $R_6L_2W_3$-D는 $R_6L_2W_3$과 비교 했을 때 더욱 높은 독성을 나타내었다. *C. albicans*의 세포막을 모방하는 vesicles로 부터의 약한 형광마크의 방출은 Lys을 함유한 펩타이드의 주요한 표적위치는 *C. albicans*의 세포막이 아니라, 세포질이라는 것을 제시한다. Confocal laser-scanning microscopy실험에서는 FITC로 표지된 Lys을 함유한 펩타이드는 세포벽과 세포막을 통과하여 세포 안으로 들어가지만 FITC로 표지된 Arg을 함유한 팹타이드는 세포막을 통과 하지 못하였다. 이상의 결과는 Arg을 함유한 펩타이드의 최종 표적위치는 *C. albicans*의 세포막이며, Lys을 함유한 펩타이드의 최종 표적위치는 세포질이라는 것을 제시한다.

PART Ⅰ

Prokaryotic selectivity and anti-inflammatory activity of short antimicrobial peptides designed from human cathelicidin antimicrobial peptide, LL-37

1. Introduction

Antimicrobial peptides (AMPs) are essential host defense molecules found in a wide variety of species ranging from bacteria, fungi, plants, amphibians, to mammals, including humans [1-5]. AMPs are now considered promising and attractive candidates for the design and development of new therapeutics due to their new modes of action to slow down the alarming trend of resistance [2, 3]. In mammals, two major classes of AMPs have been identified, the cathelicidins and defensins [6]. Cathelicidins are characterized by highly conserved N-terminal prosequence termed the "cathelin" domain and highly variable C-terminal peptide domain in which the antimicrobial activity is found [7-9]. CAMP (also known as hCAP18) is the only member of cathelicidins found in humans [10, 11]. The C-terminal end of CAMP is proteolysed to generate a small peptide of 37 amino acid residues starting with two leucine residues, so called LL-37 [12]. Like the majority of AMPs found in nature, LL-37 has an amphipathic α-helical structure and carries a positive net charge of +6 at a physiological pH [13, 14]. LL-37 has killing activity against a broad range of Gram-positive and Gram-negative bacteria, as well as fungi, in vitro [15]. Unfortunately, LL-37 is toxic to human erythrocytes [14], leukocytes and T-lymphocyte MOLT cell lines [13], probably due to hydrophobic interactions with the eukaryotic cell membrane [14].

LPS (lipopolysaccharide), also termed endotoxin, is a major constituent of the outer membranes of Gram-negative bacteria and is recognized as a key molecule in the pathogenesis of endotoxin shock associated with Gram-negative bacterial infections [16, 17]. LPS is released from the bacteria during cell division, cell death, or in particular, as a result of antibiotic treatment against bacterial infection [18]. Although the exact mechanism is not yet well understood, upon its release, LPS interacts with the LPS binding proteins (LBP), accelerating the binding of LPS to CD14, the primary receptor of LPS, which is expressed mainly on macrophages [19-21]. The LPS–LBP–CD14 complex initiates intracellular signaling by interacting with the transmembrane protein Toll-like receptor-4 (TLR-4), which activates NF-κB transcriptional factor, resulting in the

activation of genes coding for pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), nitric oxide (NO) and others [22-25]. However, an unbalanced and overwhelming production of these cytokines may lead to septic shock characterized by endothelial tissue damage, loss of vascular tone, coagulopathy, and multiple-system organ failure, frequently resulting in death [26, 27].

Recently, it has been shown that LL-37 protects the host from endotoxic shock by blocking the binding of LPS to $CD14^+$ cells, thereby suppressing the production of cytokines by these cells [28-31]. The combination of antimicrobial and LPS-neutralizing properties make LL-37 an attractive therapeutic agent for endotoxin shock and sepsis caused by Gram-negative bacterial infections [28-31]. In the present study, in order to develop novel AMPs with shorter in length, improved prokaryotic selectivity and retained LPS-neutralizing activity compared to natural LL-37, I designed and synthesized a series of amino acid-substituted analogues based on IG-19 (residues 13-31) which known as the α-helical region and the active domain for the modulation of TLR responses of LL-37 [32, 33]. To increase the resistance to proteases of our designed peptides, diastreomeric peptides composed of D- and L-amino acids and D-enantiomeric peptides were synthesized.

The prokaryotic selectivity of the peptides was investigated by examining their antimicrobial activity against Gram-positive and Gram-negative bacterial strains and their hemolytic activity against human red blood cells. The anti-inflammatory activity of the peptides were evaluated by examining inhibition of NO production, inducible nitric oxide synthase (iNOS) mRNA expression, TNF-α secretion and TNF-α mRNA expression in LPS-stimulated RAW264.7 cells, a mouse macrophage cell line. The LPS-neutralizing activity of the peptides was examined by the chromogenic *Limulus amebocyte* lysate (LAL) assay. Secondary structure of the peptides in bacterial membrane-mimicking environments was investigated by circular dichroism (CD) spectroscopy. These results will help in the design of novel short AMPs with prokaryotic selectivity and anti-inflammatory activity.

2. Materials and methods

2.1. Materials

Rink amide 4-methylbenzhydrylamine (MBHA) resin and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem-Novabiochem (La Jolla, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA; Sigma), piperidine (Merck), dicyclohexylcarbodiimide (DCC; Fluka), *N*-hydroxybenzotriazole hydrate (HOBT; Aldrich) and dimethylformamide (DMF, peptide synthesis grade; Biolab). Phosphatidylcholine (PC, from egg yolk), phosphatidylethanolamine (PE, from egg yolk), phosphatidylglycerol (PG, from egg yolk), cholesterol, lipopolysaccharide (LPS, from *Escherichia coli* O111:B4), trifluoroethanol (TFE), sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and trypsin (from bovine pancreas, EC 3.4.21.4) were purchased from Sigma Chemical Co (St. Louis, MO). DMEM and fetal bovine serum (FBS) were supplied by HyClone (SeouLin, Bioscience, Korea). RAW 264.7 cells were purchased from American Type Culture Collection (Bethesda, MD). All other reagents were of analytical grade. The buffers were prepared in double glass-distilled water.

2.2. Peptide synthesis

Peptides listed in Table 1 were prepared using the standard Fmoc-based solid-phase synthesis technique on Rink amide MBHA resin. DCC and HOBt were used as coupling reagents, and 10-fold excess of Fmoc-amino acids was added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/H_2O/thioanisole/phenol/ethanedithiol/triisopropylsilane (81.5:5:5:5:2.5:1, v/v) for 2 h at room temperature, crude peptides were repeatedly extracted with diethyl ether and purified by reverse-

phase high-performance liquid chromatography (RP-HPLC) on a preparative Vydac C_{18} column (15 μm, 20 mm × 250 mm) using an appropriate 0–90% water/acetonitrile gradient in the presence of 0.05% TFA. The final purity of the peptides (>98%) was assessed by RP-HPLC on an analytical Vydac C_{18} column (4.6 mm × 250 mm, 300Å, 5-μm particle size). The molecular masses of purified peptides were determined using matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan). Peptide concentrations were determined by amino acid analysis (Hitachi Model, 8500 A, Japan).

2.3. Antimicrobial activity (MIC)

The antimicrobial activity of the peptides against three Gram-positive bacterial strains and three gram-negative bacterial 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 Autoreader EL 800 (Bio-Tek Instruments, VT). The minimal inhibitory concentration (MIC) was defined as the minimum peptide concentration inhibited bacteria growth. Three types of gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC 1621]) and 3 types of gram-negative bacteria (*E. 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 (KRIBB).

2.4. Hemolytic activity

Fresh human red blood cells (hRBCs) were washed 3 times with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4) by centrifugation for 7 min at 1000 × g, and resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 μl of hRBC suspension [4% (v/v) in final] in PBS to a final volume of 200 μl, and incubated for 1h at 37°C. Samples were centrifuged at 1000 × g for 5 min, and hemoglobin release was monitored by measuring the supernatant absorbance at 405 nm with a Microplate ELISA Reader (Bio-Tek Instruments, VT, USA). Minimal hemolytic concentration (MHC) was defined as the minimal peptide concentration that produced 10% hemolysis. hRBCs in PBS (A_{blank}) or 0.1% Triton X-100 (A_{triton}) were used as the negative and positive controls, respectively. The hemolysis percentage was calculated according to the equation:

$$\% \text{ hemolysis} = 100 \times [(A_{sample} - A_{blank}) / (A_{triton} - A_{blank})]$$

2.5. Resistance to proteolytic digestion

Bacteria (*E. coli* and *S. aureus*) were grown overnight for 18 h at 37 °C in 10 ml of LB broth and then 10 μl of this culture was inoculated into 10 ml of fresh LB and incubated for an additional 3 h at 37 °C to obtain mid-logarithmic phase organisms. For the radial diffusion assay method, a bacteria suspension (2×10^6 CFU/ml in LB) was mixed with 0.7% agarose. The mixture was poured into a 10-cm petri dish after rapidly dispersing. Five microliters of an aqueous peptide stock solution (10 mg/mL) were added to 25 μl of 0.2 μg/ml trypsin solution in PBS, receptively, and incubated at 37 °C for 6 h. The reaction was stopped by freezing with liquid nitrogen, and then ten-microliters aliquotswere placed in each circle paper (≈ 6mmin diameter) put on the agarose plates and then incubated at 37 °C for overnight. The diameters of the bacterial clearance zones surrounding the circle paper were measured for the quantitation of inhibitory activities. For the broth microdilution assay method, aliquots (100 μl) of a bacterial suspension (2×10^6 CFU/ml in 1% peptone) were added to 100 μl of each peptide in 1% peptone (final peptide concentration: 2× MIC

for bacteria). After incubation for 18–20 h at 37 °C, bacterial growth inhibition was determined by measuring absorbance at 600 nmwith a Microplate autoreader EL 800 (Bio-Tek Instruments).

2.6. Circular dichroism (CD) spectroscopy

The circular dichroism (CD) spectra of the peptides were recorded at 25 °C using a Jasco J-715 CD spectrophotometer (Tokyo, Japan). The samples were scanned at room temperature in a capped quartz cuvette (1-mm path length) cells in the wavelength range of 190–250 nm. The spectra were recorded at a peptide concentration of 100 μg/ml in 0.1% LPS or 30mM sodium dodecyl sulfate (SDS). The mean residue ellipticity, [θ], was given in $deg \cdot cm^2 \cdot dmol^{-1}$: $[\theta] = [\theta]obs\ (MRW/10 \times l \times c)$, 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. The spectra were expressed as molar ellipticity [θ] vs. wavelength. The percentage α-helicity of the peptides was calculated as follows: $\% \ \alpha\text{-helicity} = ([\theta]_{222} - [\theta]^0_{200} / ([\theta]^{100}_{222} - [\theta]^0_{200}) \times 100$, where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm and values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, which correspond to 0 and 100% helix content at 222 nm, are estimated to be -2000 and -32000, respectively [34].

2.7. Cell culture

RAW 264.7 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum and antibiotic-antimyotic solution (100 units/ml penicillin, 100g/ml streptomycin and 25g amphotericin B) in 5% CO_2 at 37 °C. Cultures were passed every 3 to 5 days, and cells were detached by brief trypsin treatment, and visualized with an inverted microscope.

2.8. Cytotoxicity (MTT proliferation assay)

Cytotoxicity of peptides against RAW 264.7 cells was determined using the MTT assay as reported previously [35], with minor modifications. RAW 264.7 cells were seeded on 96-well microplates at a density of 2×10^4 cells/well in 150 μl DMEM 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 DMEM) were added, and the plates further incubated for 2 days. 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 2h. Absorbance at 570 nm was measured using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA). Cell survival was expressed as a percentage of the ratio of A_{570} of cells treated with peptide to that of cells only.

2.9. Nitric oxide (NO) production from LPS-stimulated RAW 264.7 cells

Nitrite accumulation in culture media was used as an indicator of nitric oxide (NO) production. Cells were plated at a density of 5×10^5 cells/ml in 96-well culture plates, and stimulated with LPS (20 ng/ml) in the presence or absence of peptides for 24 h. Isolated supernatant fractions were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Nitrite production was measured by absorbance at 540 nm, and the concentrations determined using a standard curve generated with $NaNO_2$.

2.10. Evaluation of TNF-α release from LPS-stimulated RAW264.7 cells

RAW264.7 macrophages were cultured overnight in 96-wells plate (2.5×10^5 cells/well). The

medium was then removed followed by the addition to each well of fresh DMEM supplemented with 5% of bovine serum. The cells were stimulated with LPS (20 ng/ml) in the presence or absence of peptides. Cells that were stimulated with LPS alone, and untreated cells served as controls. The cells were incubated for 24 h at 37 °C after which samples of the medium from each treatment were collected. TNF-α concentration in the samples was evaluated using a mouse TNF-α enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (ELISA, Biosource). All experiments were done in duplicate.

2.11. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW264.7 cells were plated at 5×10^5 cells/well in six-well plates and cultured overnight. Cells were stimulated for 6 h without (negative control) or with 20 ng/ml LPS in the presence or absence of peptide in DMEM supplemented with 10% bovine serum. The cells were detached from the wells and washed once with phosphate-buffered saline. Total RNA was prepared, and reverse transcribed to cDNA with oligo(dT)15 primers. The cDNA products were amplified in the presence of primers for iNOS (forward, 5'-CTGCAGCACTTGGATCAGGAACCTG-3'; reverse, 5'-GGGAGTAGCCTGTGTGCACCTGGAA-3'); TNF-α (forward, 5'-CCTGTAGCCCACGTCGTAGC-3'; reverse, 5'-TTGACCTCAGCGCTGAGTTG-3'); and β-actin (forward, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; reverse, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'). The amplification protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min.

2.12. LPS neutralizing assay

The ability of the designed peptides to neutralize or inhibit LPS was assessed using a commercially available Limulus amebocyte lysate (LAL) assay kit (Kinetic-QCL 1000 kit; BioWhittaker, Walkersville, MD, USA) [36, 37]. Briefly, 25 µl of serially diluted peptide was added in duplicate to 25 µl of *E. coli* O55:B5 LPS containing 3.0 U/ml endotoxin for 30 min at 37 °C, followed by incubation with 50 µl of amoebocyte lysate reagent for 10 min. Absorbance at 405 nm was measured 10 and 16 min after the addition of 100 µl of the chromogenic substrate, Ac-Ile-Glu-Ala-Arg-p-nitroanilide. The amount of non-bound LPS was extrapolated from a standard curve, and percentage inhibition calculated as: [(amount of free LPS in control samples) − (amount of free LPS in test samples)] × 100 /amount of free LPS in control samples.

3. Results

3.1. Peptide design

To develop short AMPs with more improved prokaryotic selectivity and retained LPS-neutralizing activity compared to natural LL-37 and investigate the effects of various structural parameters of the peptide on LPS-neutralizing activity, I designed and synthesized a series of short analogs based on IG-19 (residues 13-31 of LL-37). Amino acid sequences of IG-19 and its analogs generated in this study are summarized in Table 1. To design each peptide sequence, I used the α-helical wheel diagrams shown in Fig. 1. As shown in its α-helical wheel diagram, the starting molecule, IG-19 adopted an amphipathic α-helical structure by converging the hydrophobic residues to one side and the hydrophilic residues to the other side of the helical axis. The analogs, a1, a2, a3 and a4 were designed by increasing of net positive charge and decreasing hydrophobicity by introducing more lysines. The analogs, a5, a6, a7, a8 and a9 were generated by increasing of net positive charge or/and hydrophobicity by introducing more lysines or/and leucines.

Furthermore, to increase the membrane interaction of peptides, I synthesized three Trp-substituted a4 analogs (a4-W1 and a4-W2), which Phe^{15} or Phe^{5} of a4 is replaced by Trp, respectively (Fig. 2). In addition, to provide endogenous proteases of these peptides, the diastereomeric peptides (a4-W1-D and a4-W2-D) and D-enantiomeric peptides (a4-W1-E and a4-W2-E) were designed and synthesized (Table 1).

3.2. Hydrophobicity of peptides

The hydrophobicity of peptides was assessed by measuring the RP-HPLC retention time (Table 1). The retention time of peptides on a reverse-phase matrix was reported to be related to peptide hydrophobicity [38].

3.3. Circular dichroism (CD) spectroscopy

The secondary structures of the all peptides were determined in 0.1 % LPS solution (Fig. 3). All of the peptides exhibited a predominant α-helical structure characterized by double minima at 208 and 222 nm in the presence of LPS, except for a9. D-enantiomeric peptides (a4-W1-E and a4-W2-E) had a left-handed α-helical structure with a major conformer (Fig. 3) The percent α-helicity of the peptides in 0.1 % LPS solution was calculated from ellipiticity at 222 nm (Table 1).

3.4. Antimicrobial (MIC) and hemolytic (MHC) activities

I examined the antimicrobial activities of these peptides against a representative set of bacterial cells, including three Gram-negative bacteria (*E. coli, P. aeruginosa and S. typhimurium*) and three Gram-positive bacteria (*B. subtilis, S. epidermidis* and *S. aureus*). IG-19 and all its analogs were likewise similarly effective when assessed using the geometric mean (GM) of the MIC values (3.0~9.3 μM) from selected all microbial strains, as an overall measure of the antimicrobial activity of the peptides (Table 2). The cytotoxicity of the peptides to mammalian cells of peptides by measuring their hemolytic activity toward human red blood cells (h-RBCs) was measured. For a quantitative measure of the hemolytic activity of the peptides, I introduced the minimal hemolytic concentration (MHC) defined as the peptide concentration that produces 10% hemolysis (Table 3).

3.5. Therapeutic index (TI)

The therapeutic potential of peptide antimicrobial drugs lies in the prokaryotic selectivity to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. The prokaryotic selectivity of the peptides is defined by the concept of the therapeutic index (TI) as

a measure of the relative safety of the drug [39-46]. The TI of each peptide was calculated as the ratio of the MHC value to the GM (geometric mean of MICs against six selected microorganisms). When there was significant no hemolysis at the highest concentration tested (200 μM), 400 μM was used for the TI calculation, since the test was carried out by two-fold serial dilution. A high TI value is thus an indication of two preferred characteristics of the peptide: a high MHC (low hemolytic activity) and a low MIC (high antimicrobial activity). The TI values for each peptide were shown in Table 3.

3.6. Cytotoxicity toward RAW264.7 cells

The cytotoxicity of the peptides in RAW264.7 macrophage cells was evaluated by a standard MTT assay, which demonstrates active energization of cells and is conventionally used as a measure of cell viability. As shown in Fig. 4, IG-19 and LL-37 were non-toxic to RAW264.7 cells at 50μM and 25μM, respectively, with cell viability above 90% at these concentrations. All of the peptides were non-toxic until 6.25μM, except for I8. Therefore, all experiments used RAW264.7 macrophage cells were performed at concentrations less than 5 μM.

3.7. Inhibition of nitric oxide (NO) production in LPS-stimulated RAW264.7 cells

Nitric oxide (NO) is an important signaling molecule that is primarily involved in promoting the inflammatory response. To assess the potential anti-inflammatory activity of the peptides, I indirectly measured peptide inhibition of NO production in 20 ng/ml of LPS-stimulated RAW264.7 macrophages by quantifying nitrite concentration. Cells were treated with or without 20 ng/ml LPS at the fixed concentration (0.5 μM) of each peptide (Fig. 5). The percent inhibition of NO production of each peptide at 0.5 μM was summarized in Table 3. Similar to LL-37, a5, a6, a7, a8, a9, a4-W1, a4-W1-E and a4-W2-E inhibited significantly NO production in LPS-simulated

RAW264.7 macrophages at 0.5 μM (Fig. 5). Also, a4-W1, a4-W1-E, and a4-W2-E in various concentrations of 0.25, 0.5, 1.0 and 2.5 μM showed inhibitory activity of NO production comparable to that of LL-37 (Fig. 6).

3.8. Inhibition of TNF-α release from LPS-stimulated RAW264.7 cells

TNF-α is one of the first pro-inflammatory cytokines secreted by LPS-stimulated immune cells. To explore the effect of the peptides on LPS-induced TNF-α secretion, the macrophages were stimulated with 20 ng/ml LPS. The ability of the peptides to detoxify LPS was examined by monitoring the concentration of the secreted TNF-α in the presence of the peptides. The percent inhibition of TNF-α release of each peptide at 0.5 μM was summarized in Table 3. Similar to LL-37, a5, a6, a7, a8, a9, a4-W1, a4-W1-E and a4-W2-E inhibited significantly TNF-α release in LPS-simulated RAW264.7 macrophages at 5.0 μM (Fig. 7). As shown in Fig. 8, a4-W1, a4-W1-E, and a4-W2-E showed relatively lower inhibitory activity of NO production at the concentration of 0.5, 1.0 and 2.5 μM, in comparison to LL-37.

3.9. Inhibition of LPS-induced iNOS gene expression

LPS induces iNOS protein expression in macrophages, which correlates with NO production; the effects of the peptides on LPS-induced iNOS gene expression (LPS: 20 ng/ml) induced in LPS-stimulated RAW264.7 macrophage cells were examined over a period of 6 h (Fig. 9). Similar to LL-37, a4-W1, a4-W2 a4-W1-E and a4-W2-E significantly inhibited iNOS gene expression at 1.0 μM. In contrast, a4, a4-W1-D and a4-W2-D did not completely inhibit iNOS mRNA expression at 1.0 μM.

3.10. Resistance to proteolytic digestion

Poor protease stability severely limits the therapeutic application of AMPs [47, 48]. We therefore examined the susceptibility of peptides to trypsin. Trypsin specifically catalyzes the hydrolysis of the C-terminal amide bonds of Lys and Arg, making the enzyme an ideal tool in the present study, since the synthesized peptides possess several Lys residues. The peptides were pretreated with trypsin and their residual antimicrobial activity was assayed using the radial diffusion assay method and the broth microdilution assay method. As shown in Fig. 10, trypsin treatment of a4, a4-W1, a4-W2 and a4-W3 completely abolished antimicrobial activities against both *E. coli* and *S. aureus*. In contrast, the antimicrobial activity of a4-W1-D, a4-W2-D, a4-W1-E and a4-W2-E was completely preserved after trypsin treatment.

3.11. Neutralization of LPS activity by the peptides in vitro

The chromogenic LAL assay is an extremely sensitive indicator in the presence of free, non-neutralized LPS [49], allowing the detection of free LPS at the pg/ml level. LAL assay results are generally accepted as representing the ability of a molecule to neutralize LPS. Thus, the ability of the peptides to neutralize LPS from *E. coli* 0111:B4 was determined by LAL assay (Fig. 11). LAL assays were conducted at the LPS concentration of 3 EU/ml, with four different concentrations (0.5, 1.0, 2.5 and 5.0 μM) of peptides. Similar to polymyxin B, a positive control peptide, almost of the peptides inhibited ≥90% endotoxin at 5.0 μM. In contrast, both a4-W1-D and a4-W2-E displayed a weak inhibitory activity, only 15% ≤ at 5.0 μM.

Table 1. Amino acid sequence, net charge, RP-HPLC retention time and % α-helicity of IG-19 and its analogs.

Peptides	Amino Acid Sequences	Net charge	RT-HPLC[a] (min)	% α-helicity (0.1% LPS)
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+6	32.07	91.2
IG-19	IGKEFKRIVQRIKDFLRNL	+5	29.57	65.8
a1	IGK**K**FKRIVQRIKDFLRNL	+7	27.16	86.8
a2	IGK**K**FKRIVQRIK**K**FLRNL	+9	25.31	63.7
a3	IGKKFKRIVQRIKKFLRKL	+10	24.14	60.1
a4	IGKKFKRIVKRIKKFLRKL	+11	22.20	51.2
a5	IGKLFKRIVQRIKKFLRNL	+7	29.81	74.5
a6	IGKLFKRIVQRILKFLRNL	+7	35.25	100.0
a7	IGKLFKRIVKRILKFLRKL	+9	30.31	52.4
a8	ILKLFKRIVKRILKFLRKL	+9	31.86	32.2
a9	ILKLFKRIVKLILKFLRKL	+8	37.57	0.0
a4-W1	IGKKFKRIVKRIKKWLRKL	+11	22.54	76.1
a4-W2	IGKKWKRIVKRIKKFLRKL	+11	22.96	70.3
a4-W1-D	IGkKFKrIVkRIkKWLrKL	+11	18.31	27.9
a4-W2-D	IGkKWKrIVkRIkKFLrKL	+11	19.01	29.6
a4-W1-E	igkkfkrivkrikkwlrkl	+11	22.47	70.4
a4-W2-E	igkkwkrivkrikkflrkl	+11	22.99	77.2

[a] The peptides were eluted using a 60-min linear gradient of acetonitrile (0%) and water (90%) containing 0.1% trifluoroacetic acid (v/v).

Table 2. Minimal inhibitory concentrations of IG-19 and its analogs on different microbial strains

Peptide	Minimal Inhibitory Concentration (MIC: μM)					
	Gram-negative bacteria			Gram-positive bacteria		
	E. coli	*P. aeruginosa*	*S. typhimurium*	*B. subtilis*	*S. epidermidis*	*S. aureus*
IG-19	8	16	8	4	4	4
a1	8	8	2	2	2	4
a2	8	8	8	2	2	4
a3	8	8	4	2	2	4
a4	8	8	2	2	4	4
a5	8	8	4	4	4	8
a6	8	8	4	2	8	8
a7	8	8	4	2	4	4
a8	8	8	8	4	4	8
a9	8	8	8	2	8	4
a4-W1	4	4	2	2	4	2
a4 -W2	4	4	2	2	4	4
a4 -W1-D	2	2	2	2	4	4
a4 -W2-D	4	4	2	2	4	4
a4 -W1-E	4	4	2	2	4	4
a4 -W2-E	4	4	2	2	4	4
LL-37	8	8	4	8	8	4

Table 3. Therapeutic index (prokaryotic selectivity) and anti-inflammatory activity of IG-19 and its analogs

Peptide	GM (μM)[a]	MHC (μM)[b]	Therapeutic Index (TI)[c] (MHC/GM)	% NO inhibition[d]	% TNF-α inhibition[e]
IG-19	7.3	21.6	2.9	43.1±6.4	47.7±4.9
a1	4.3	13.8	3.2	40.0±8.4	38.4±0.9
a2	5.3	11.2	2.1	22.0±5.4	9.1±1.7
a3	4.7	23.7	5.0	27.8±2.7	9.9±3.1
a4	4.7	33.2	7.1	31.5±2.0	33.5±1.4
a5	6.0	3.0	0.5	78.3±3.0	82.8±1.2
a6	6.3	1.2	0.2	67.8±2.0	69.6±0.7
a7	5.0	1.1	0.2	84.1±1.6	74.9±2.4
a8	6.7	1.6	0.2	79.3±7.4	79.8±1.6
a9	6.3	0.7	0.1	73.9±3.0	74.5±2.4
a4-W1	3.0	8.4	2.8	82.4±10.1	69.8±3.0
a4 -W2	3.3	9.2	2.8	47.5±8.4	26.1±3.5
a4 -W1-D	2.7	200 <	148.1	12.9±8.1	10.7±3.4
a4 -W2-D	3.3	200 <	121.2	4.70±5.4	20.4±2.7
a4 -W1-E	3.3	10.8	3.3	83.1±7.4	72.6±0.7
a4 -W2-E	3.3	11.6	3.5	64.4±2.0	66.1±1.4
LL-37	6.7	6.7	1.0	87.1±3.7	83.3±1.8

[a] The geometric mean (GM) of the MIC values against 6 bacterial strains.
[b] The MHC (minimal hemolytic concentration) is the peptide concentration causing 10% hemolysis.
[c] The ratio of the MHC (μM) to the GM (μM).
[d] % inhibition of NO production at the peptide concentration of 0.5 μM
[e] % inhibition of TNF-α release at the peptide concentration of 5.0 μM

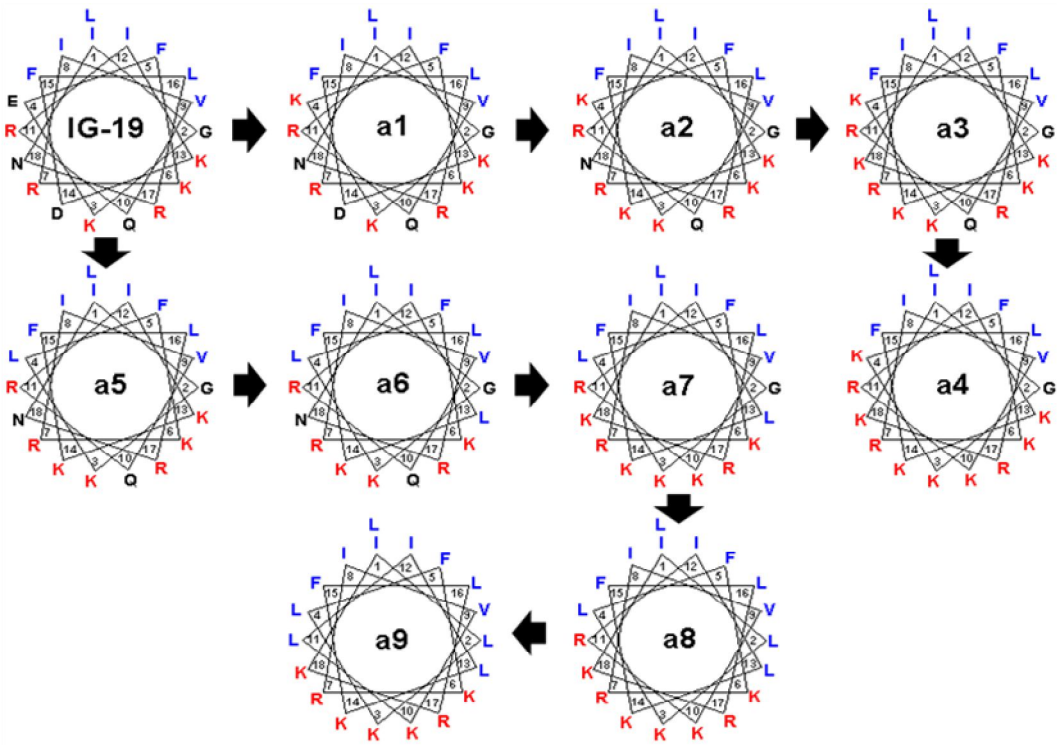

Figure 1. Helical wheel diagrams for IG-19 and its analogs (a1-a9) designed in this study. The peptide name is presented in the middle of each diagram. The order of design is depicted by the flow of the arrows.

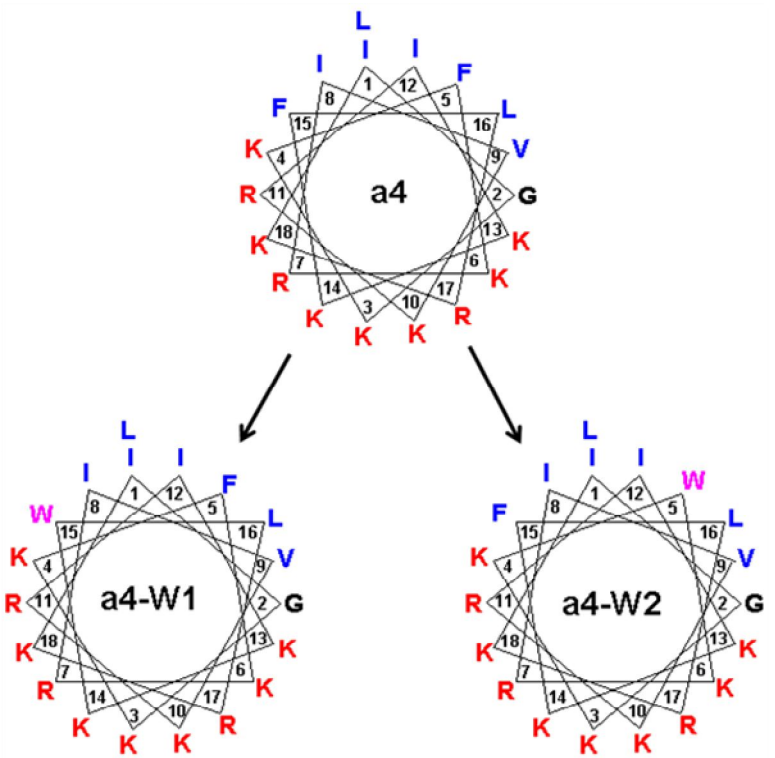

Figure 2. Helical wheel diagrams for Trp-substituted a4 analogs. The peptide name is presented in the middle of each diagram.

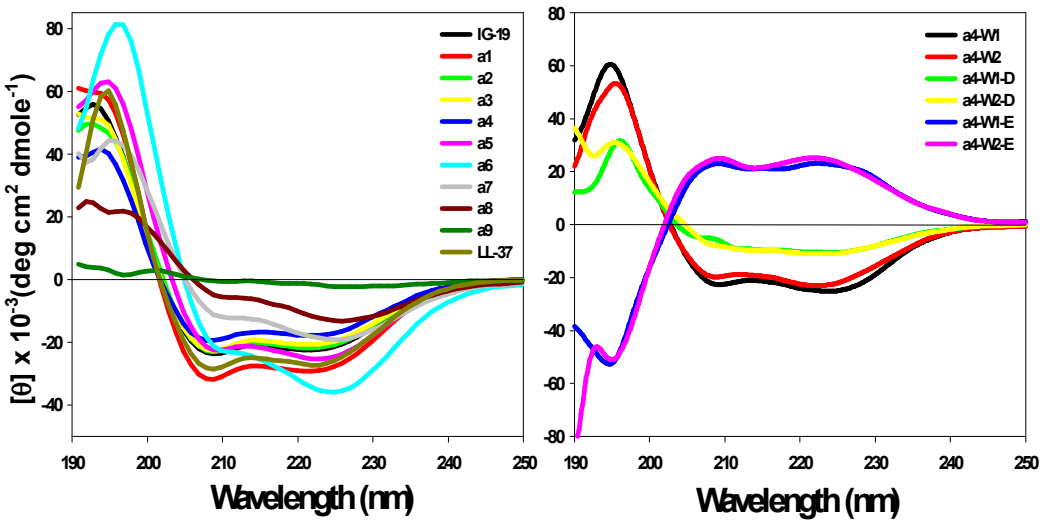

Figure 3. Circular dichroism (CD) spectra of the peptides in 0.1 % LPS suspension.

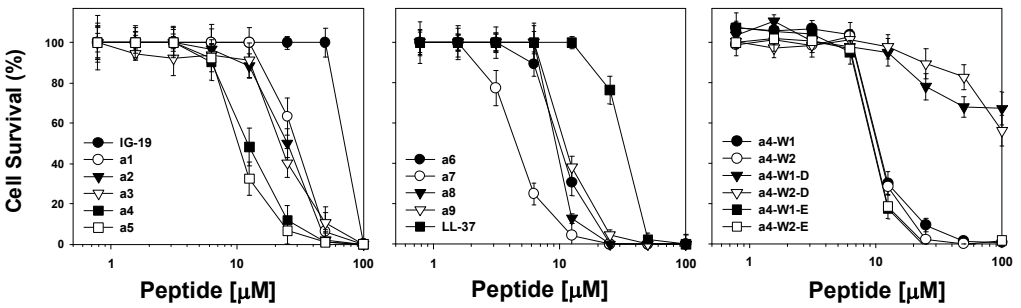

Figure 4. Cytotoxicity of peptides against macrophage-derived RAW264.7 cells.

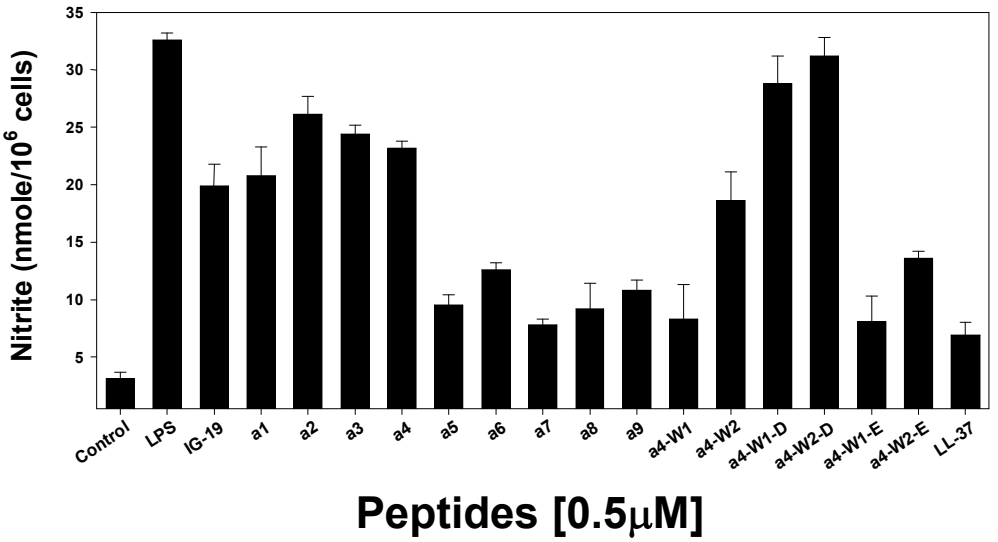

Figure 5. Inhibitory effects of peptides on LPS-induced nitric oxide (NO) production in RAW264.7 cells. RAW264.7 cells (5×10^5 cells/ml) were treated with 20 ng/ml LPS in the absence or presence of the peptides (0.5 μM) for 24h. The cell culture media were then collected, and the amount of nitrite released was measured. The error bars represent standard deviations of the mean determined from three independent experiments.

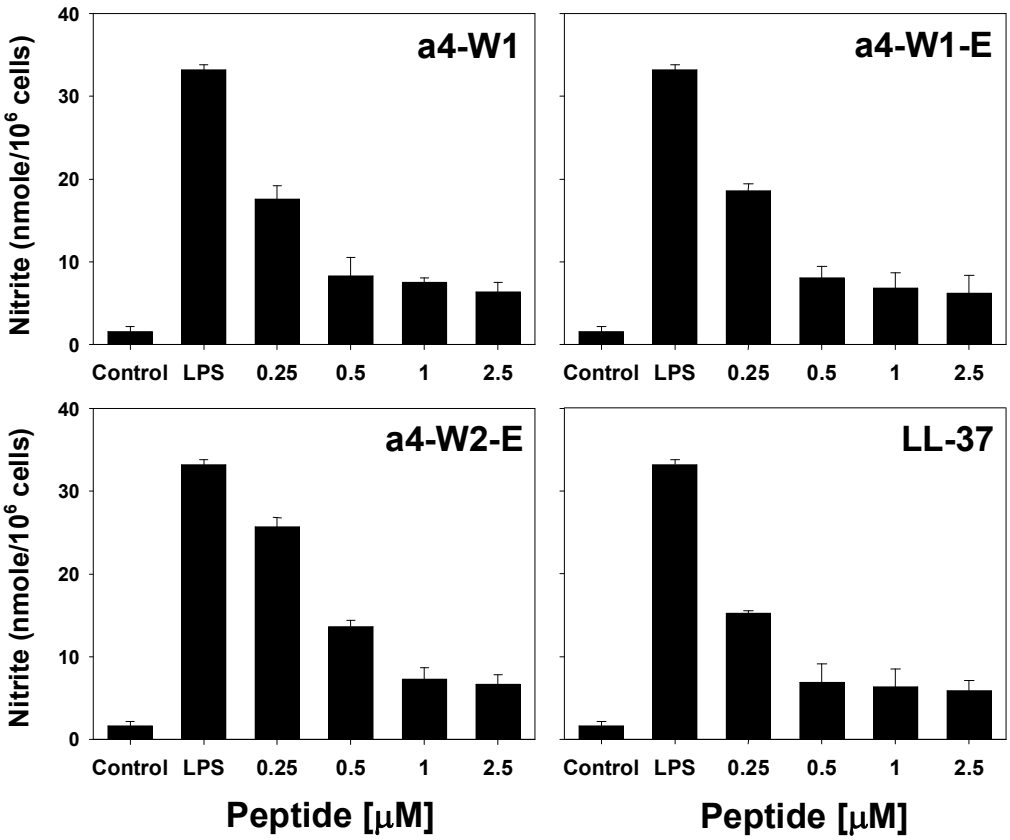

Figure 6. Inhibitory effects of peptides on LPS-induced nitric oxide (NO) production in RAW264.7 cells. RAW264.7 cells (5×10^5 cells/ml) were treated with 20 ng/ml LPS in the absence or presence of various concentrations (0.25 μM, 0.5 μM, 1.0 μM and 2.5 μM) of the peptides for 24h. The cell culture media were then collected, and the amount of nitrite released was measured. The error bars represent standard deviations of the mean determined from three independent experiments.

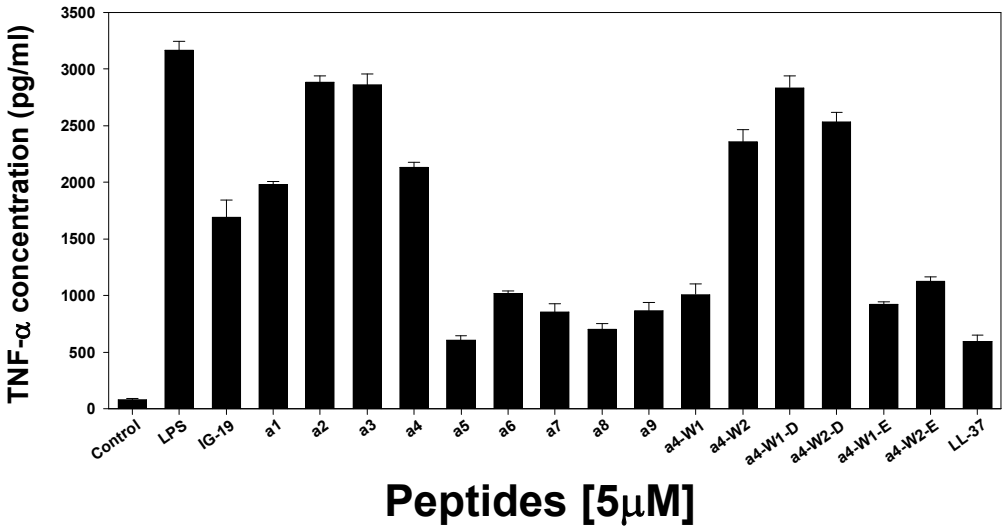

Figure 7. Effects of the peptides on LPS-induced TNF-α secretion by macrophages. RAW264.7 cells (5×10^5 cells/ml) were treated with 20 ng/ml LPS in the absence or presence of the peptides (5.0 μM) for 24h. After incubation, the TNF-α concentration in the cell medium was evaluated using a mouse TNF-α ELISA Kit.

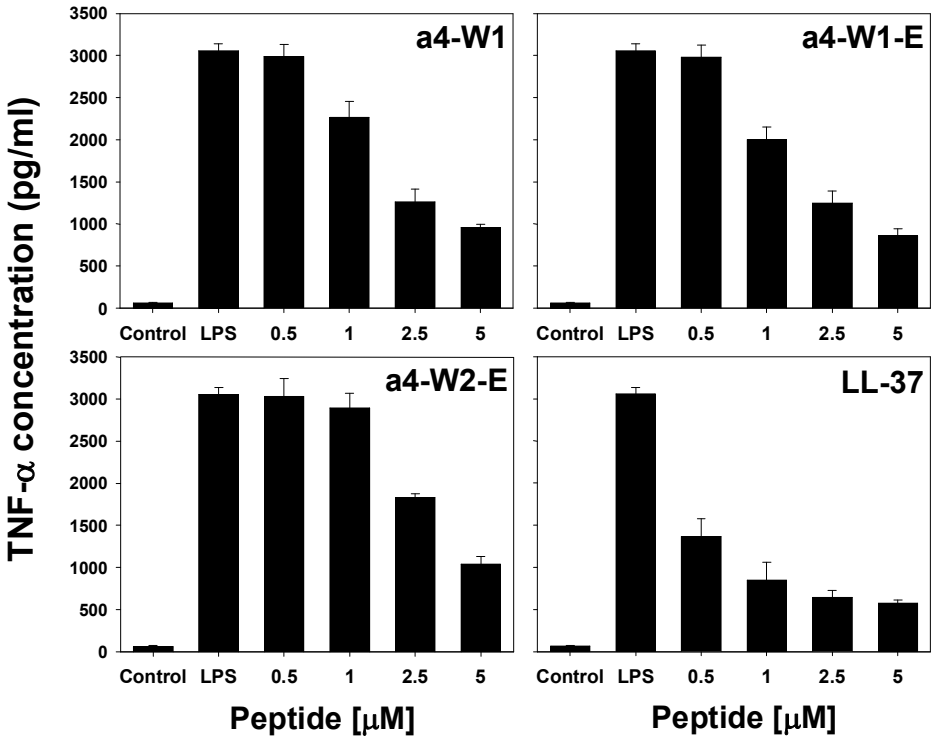

Figure 8. Effects of the peptides on LPS-induced TNF-α secretion by macrophages. RAW264.7 cells (5×10^5 cells/ml) were treated with 20 ng/ml LPS in the absence or presence of various concentrations (0.25 μM, 0.5 μM, 1.0 μM and 2.5 μM) of the peptides for 24h. After incubation, the TNF-α concentration in the cell medium was evaluated using a mouse TNF-α ELISA Kit.

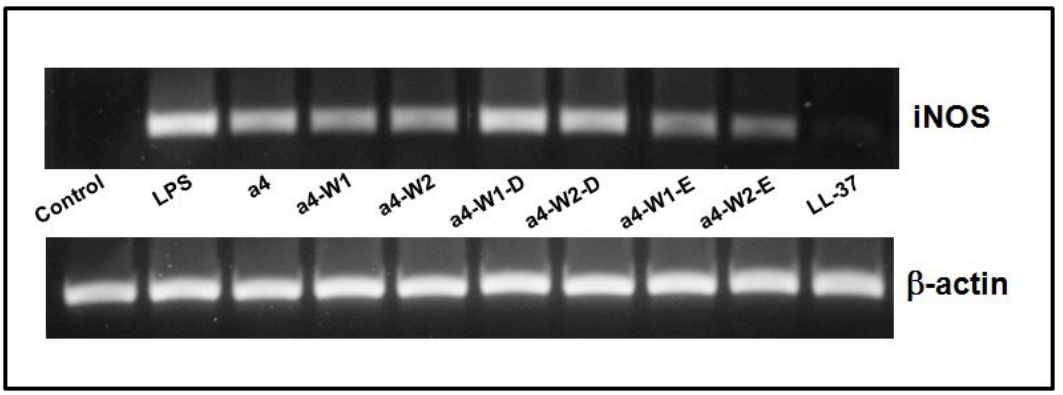

Figure 9. Inhibitory effects of peptides on LPS-induced iNOS mRNAs expression in RAW264.7 cells. RAW264.7 cells (5×10^5 cells/well) were incubated with peptides (1.0 μM) in the presence of LPS (20 ng/ml) for 8 h. Total RNA was isolated and analyzed for expression of iNOS mRNA by RT-PCR. One of two representative experiments is shown.

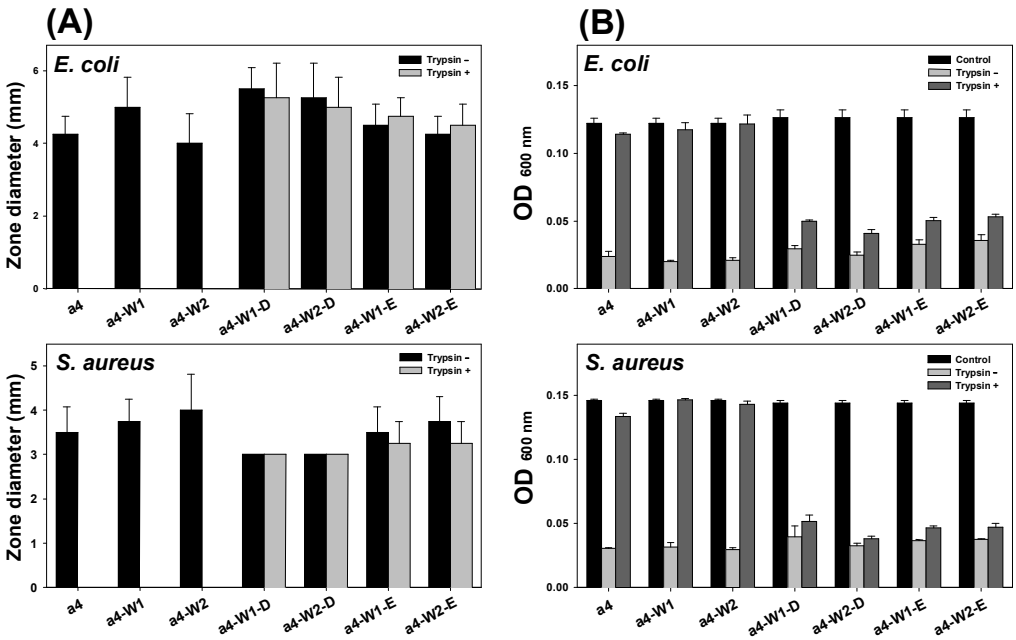

Figure 10. Inhibition of antimicrobial activity of the peptides by trypsin assessed using the radial diffusion assay (A) and broth microdilution assay (B) methods.

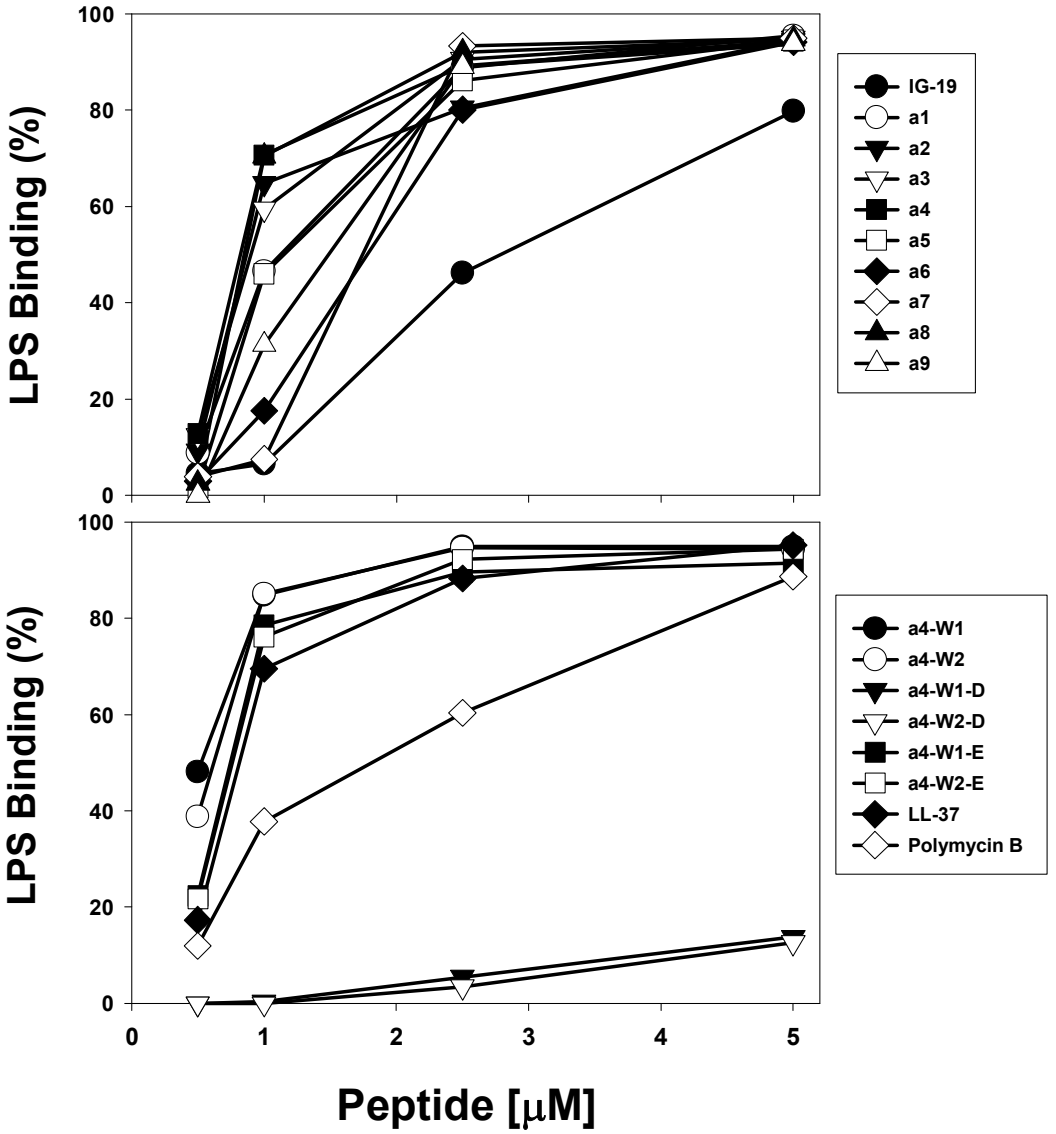

Figure 11. LPS-neutralizing activity of the peptides. *E. coli*-derived LPS (*E. coli* O111:B4 LPS) (3.0 EU/ml endotoxin) was incubated with various concentrations of each peptide for 30 min, and

the amount of free LPS was determined using the Limulus amebocyte lysate assay.

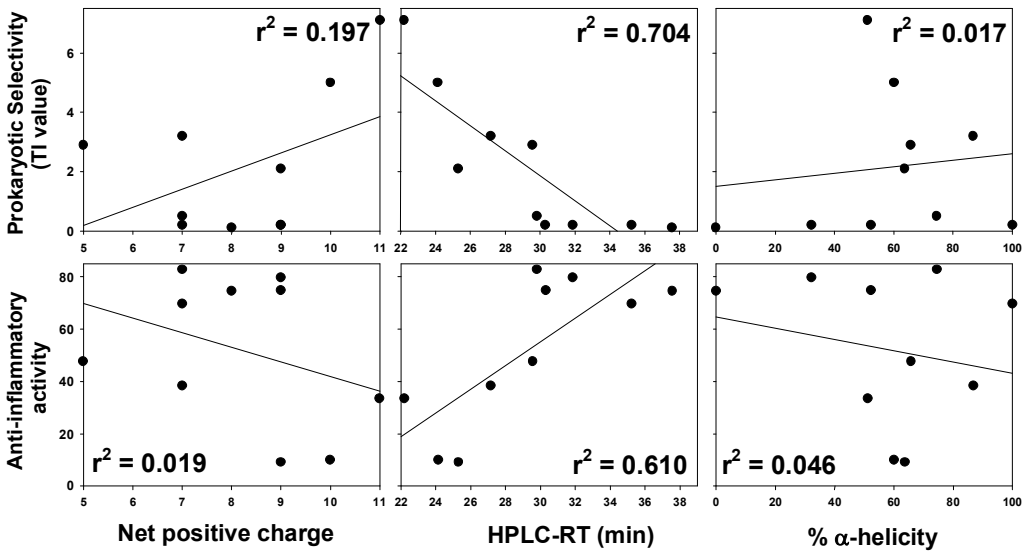

Figure 12. Relationships of net positive charge, hydrophobicity or %-α helicity of IG-19 and its analogs (a1–a9) between prokaryotic selectivity or LPS-neutralizing activity. The line was fitted by the linear regression. Each point represents one peptide.

4. Discussion

AMPs have been classified according to their prokaryotic selectivity: (a) prokaryotic selective peptides (e.g., magainin, cecropin, and defensin), which selectively have killing activity against bacterial cells; and (b) non-prokaryotic selective peptides (e.g., melittin and pardaxin), which display killing activity against both bacterial and mammalian cells. Many structure–activity studies of cationic AMPs have focused on developing prokaryotic selective peptides for use as antimicrobial drugs [50-53].

LPS released from Gram-negative bacteria during sepsis causes septic shock by inducing the production of high concentrations of systemic pro-inflammatory cytokines and NO [54]. Sepsis is the major cause of mortality in the intensive care unit, accounting for 200,000 deaths every year in the United States alone [55]. Therefore, effective AMPs should not only exert prokaryotic selectivity but also have the ability to sequester LPS and ameliorate its toxicity. Recently, in addition to its potent antimicrobial activity, LL-37 and its analogs were reported to have a powerful protective action against endotoxin shock by the direct blocking of LPS to macrophage cells, thereby suppressing the production of cytokines by these cells [29-33]. Therefore, in this study, I attempted to develop novel AMPs with shorter in length and more improved prokaryotic selectivity and retained anti-inflammatory activity compared to natural LL-37 by synthesizing a series of Lys and/or Leu-substituted-analogs based on residues 13-31 (IG-19) of LL-37.

To determine the prokaryotic selectivity of designed peptides, I calculated their therapeutic index (TI) (MHC-to-MIC ratio), which is a widely accepted measure of prokaryotic selectivity of antimicrobial agents. Larger values in TI indicate greater prokaryotic selectivity. Among our designed peptides, a4 showed the highest prokaryotic selectivity (a4 had TI enhanced 7.1-fold over that of LL-37). However, a4 exhibited much lower anti-inflammatory activity (as reflected by the inhibition of LPS-stimulated NO production or TNF-α secretion) compared to natural LL-37.

It has been reported that the net positive charge, hydrophobicity and α-helicity of α-helical AMPs mainly involved in their prokaryotic selectivity and anti-inflammatory activity [46, 56]. Thus, the relationships of the net positive charge, the hydrophobicity (as reflected by the retention time required to elute the peptide from the hydrophobic C_{18} column) or the % α-helicity of the peptides [IG-19 and its analogs (a1–a9)] between their prokaryotic selectivity (TI value) or anti-inflammatory activity (as reflected by the percentage of inhibition of LPS-stimulated TNF-α secretion at 5.0 μM) was investigated. There was a significant linear correlation between the hydrophobicity of the peptides and their TI value (r^2=0.704) or anti-inflammatory activity (r^2=0.610) (Fig. 12). Among these analogs, a5, a6, a7, a8 and a9 with much higher hydrophobicity than a4 had potent anti-inflammatory activity comparable to that of LL-37, but these peptides showed poor prokaryotic selectivity (Table 3). Collectively, these results indicated that the appropriate hydrophobicity of the peptides to exert prokaryotic selectivity and anti-inflammatory activity is of great importance.

Some studies suggested that AMPs containing Trp display more potent antimicrobial activity than those with either Phe or Tyr. The bulkier Trp side chain may ensure more efficient interaction with membrane surfaces, allowing peptides to partition in the bilayer interface [57, 58]. Therefore, in order to retain the prokaryotic selectivity and enhanced anti-inflammatory activity of a4, we designed and synthesized Trp-substituted analogs (a4-W1 and a4-W2), which Phe^{15} and Phe^{5} of a4 is replaced by Trp, respectively. The single Trp residue of a4-W1 and a4-W2 is located in the amphipathic interface between the hydrophilic ending side and the hydrophobic starting side and the hydrophobic face, in their α-helical wheel projection, respectively (Figure 2). Both a4-W1 and a4-W2 have the same net positive charge of +11 and nearly same the hydrophobicity and the percent α-helicity (Table 1). These peptides show the same prokaryotic selectivity (TI value: 2.8), but a4-W2 displays higher anti-inflammatory activity, when assessed by the percentage of inhibition of LPS-stimulated NO production or TNF-α secretion, compared to a4-W1 (Figures 5 and 7). These results suggested that the effective site for the Trp-substitution in designing novel

AMPs having higher anti-inflammatory activity without a significant reduction in prokaryotic selectivity is the amphipathic interface between the hydrophilic ending side and the hydrophobic starting side in the helical wheel projection and other important parameters of AMPs may be involved in their anti-inflammatory activity, as well as their net positive charge and hydrophobicity.

A major obstacle to the application of AMPs as human therapeutics is the susceptibility of these peptides to degradation by endogenous proteases in body fluids. Of great concern are trypsin-like proteases that are rich in the body fluids and are selective for basic residues [59, 60]. To provide the stability to proteolytic digestion of peptides, I designed and synthesized diastereomeric peptides (a4-W1-D and a4-W2-D) with D-amino acid substitution at positions 3, 7, 10, 13 and 17 of a4-W1 and a4-W2, respectively and D-enantiomeric peptides (a4-W1-E and a4-W2-E) of a4-W1 and a4-W2, respectively. The susceptibility of these diastereomeric and D-enantiomeric peptides to tryptic digestion was examined by the radial diffusion and the microdilution methods. After tryptic digestion, these diastereomeric and D-enantiomeric peptides preserved their antimicrobial activity almost completely. In addition, the diastereomeric peptides (a4-W1-D and a4-W2-D) exhibited the best prokaryotic selectivity and protease resistance, but much low anti-inflammatory activity. D-enantiomeric peptides (a4-W1-E and a4-W2-E) showed prokaryotic selectivity, anti-inflammatory activity and protease resistance.

In conclusion, a4-W1, a4-W1-E and a4-W2-E with more improved prokaryotic selectivity and retained anti-inflammatory activity compared to parental LL-37 could serve as the templates for the development of antimicrobial agents for the treatment of sepsis, as well as microbial infection.

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PART Ⅱ

Effect of the position of the disulphide bond on salt resistance and LPS-neutralizing activity of the α-helical homo-dimeric model antimicrobial peptides

1. Introduction

Antimicrobial peptides (AMPs) can be categorized into 4 major classes on the basis of their sequence and structural characteristics: (i) amphipathic α-helical linear peptides, (ii) linear peptides presenting a cyclic moiety formed by a disulphide bond at the C terminus, (iii) β-hairpin peptides stabilized by 2 or more disulphide bridges, and (iv) certain amino acid-rich linear peptides such as Pro, Arg, or Trp. The first class includes several hetero- or homodimeric AMPs such as halocidin [1], distinctin [2], dicynthaurin [3], cathelicidin CAP11 [4] and PMAP-36 [5], consisting of 2 peptide chains linked by a disulphide bond. These molecules do not share any sequence homology. These dimeric AMPs have shown a broad spectrum of antimicrobial activity, showing a remarkable range of effects on gram-positive and gram-negative bacteria, yeast, and fungi.

Considering the position of the disulphide bond, halocidin and dicynthaurin, which are isolated from the hemocytes of the tunicate, *Halocynthia aurantium*, have a disulphide bond located near the N-terminus and in the central portion of the molecules, respectively. Distinctin from tree frog *Phyllomedusa distincta* and cathelicidin CAP 11 from guinea pig neutrophils have a disulphide bond near the C-terminal portion of the molecules. A disulphide bond of PMAP-36 from pig myeloid is positioned at the C-terminus.

Salt (NaCl is the predominant salt *in vivo*) sensitivity of cationic AMPs is a major obstacle in their development as novel therapeutic agents. Although AMPs exhibit significant antibacterial activity *in vitro*, many peptides seem to lose this activity under physiological salt conditions. Salt sensitivity has been observed in several AMPs, including β-defensins, cecropins, indolicidins, gramicidins, bactenecins and magainins [6–8]. However, not all peptides are salt sensitive, and some peptides show potent salt-insensitive antimicrobial activities (e.g. clavanin, tachyplesins and polyphemusins) [8, 9]. It is possible to develop synthetic α-helical peptides that substantially vary in activity and salt resistance by altering peptide hydrophobicity, amphipathicity, charge and degree

of α-helicity [10].

Recent studies have demonstrated that in addition to their antimicrobial activities, several AMPs including human LL-37, rabbit CAP18, sheep SMAP-29, bactenecin, indolicidin and BMAP-27, have the potential to inhibit lipopolysaccharide (LPS)-induced cellular cytokine and/or nitric oxide (NO) release by directly binding to the LPS or by blocking the binding of LPS to the LPS-binding protein (LBP) [11,12]. These properties render these peptides attractive drug candidates for the treatment of endotoxin shock and sepsis caused by infection with gram-negative bacteria.

In the present study, to investigate the effects of the position of a disulphide bond located in homo-dimeric α-helical AMPs on the salt resistance and LPS-neutralizing activity, I designed and synthesized an ideal amphipathic α-helical 11-meric model peptide ($K_6L_4W_1$) composed of 6 lysine molecules, 4 leucine molecules and 1 tryptophan molecule and its 3 homo-dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M and di-$K_6L_4W_1$-C) with a disulphide bond at the N-terminus, the central position and the C-terminus of the molecules, respectively. The antimicrobial activity and bactericidal kinetics of these newly designed peptides against gram-positive and gram-negative bacterial strains were examined in the presence or absence of physiological level of NaCl. The haemolytic activity of these peptides against human erythrocytes was examined. Next, I investigated the mode of bactericidal action of the peptides by measuring their potential to cause the leakage of a fluorescent dye from lipid vesicles, the depolarization of the cytoplasmic membrane potential to *Staphylococcus aureus*. Furthermore, the LPS-neutralizing activity of these peptides was established by examining the inhibition of NO release in LPS-induced mouse macrophage RAW264.7 cells. Taken together, our results will help in designing salt-resistant α-helical homo-dimeric AMPs with potent LPS-neutralizing and antimicrobial activities.

2. Materials and methods

2.1 Materials

Rink amide 4-methylbenzhydrylamine (MBHA) resin and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem-Novabiochem (La Jolla, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA; Sigma), piperidine (Merck), dicyclohexylcarbodiimide (DCC; Fluka), *N*-hydroxybenzotriazole hydrate (HOBT; Aldrich) and dimethylformamide (DMF, Biolab). Lipopolysaccharide (LPS, from *Escherichia coli* O111:B4), egg yolk L-α-phosphatidylethanolamine (EYPE), egg yolk L-α-phosphatidyl-DL-glycerol (EYPG), and calcein were purchased from Sigma Chemical Co (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by HyClone (SeouLin, Bioscience, Korea). RAW264.7 cells were purchased from American Type Culture Collection (Bethesda, MD). All other reagents were of analytical grade.

2.2 Peptide synthesis

Peptides listed in Table 1 were prepared using the standard Fmoc-based solid-phase synthesis technique on Rink amide MBHA resin. DCC and HOBt were used as coupling reagents, and 10-fold excess of Fmoc-amino acids was added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/H_2O/thioanisole/phenol/ethanedithiol/triisopropylsilane (81.5:5:5:5:2.5:1, v/v) for 2 h at room temperature, crude peptides of monomeric peptide, $K_6L_4W_1$ and corresponding linear peptides (CKLKKLWKKLLK-NH_2, KLKKLWCKKLLK-NH_2 and KLKKLWKKLLKC-NH_2) of three dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M and di-$K_6L_4W_1$-C) were repeatedly extracted with diethyl ether and purified by RP-HPLC on a preparative Vydac C_{18} column (15 μm, 20 × 250 mm)

using an appropriate 0–90% water/acetonitrile gradient in the presence of 0.05% TFA. The molecular masses of purified linear peptides were determined using MALDI-TOF MS (Shimadzu, Japan).

2.3 Antimicrobial activity (MIC)

All bacterial strains were supplied from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The antimicrobial activity of the peptides against three gram-positive bacterial strains and three gram-negative bacterial 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 with 0 or 150 mM NaCl 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 autoreader EL 800 (Bio-Tek Instruments, VT). The minimal inhibitory concentration (MIC) was defined as the minimum peptide concentration inhibited bacteria growth.

2.4 Bactericidal kinetics

The kinetics of the peptides' bactericidal activity was assessed using *E. coli* (KCTC 1682) and *S. aureus* (KCTC 1621) at MIC × 2 in the presence of 0 or 150 mM NaCl, as described in a previous study [23]. The initial density of the cultures was approximately 1×10^5 CFU/ml. After 0, 10, 30 or 60 min of exposure to the peptides at 37°C, 50 μl aliquots of serial 10-fold dilutions (up to 10^{-3}) of the cultures were plated onto Luria-Bertani (LB) agar plates to obtain viability counts. Colonies were counted after incubation for 24 h at 37 °C.

2.5 Hemolytic activity

Fresh human red blood cells (hRBCs) were washed 3 times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.4) by centrifugation for 7 min at 1000 × g, and resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 μl of hRBC suspension [4% (v/v) in final] in PBS to a final volume of 200 μl, and incubated for 1h at 37°C. Samples were centrifuged at 1000 × g for 5 min, and hemoglobin release was monitored by measuring the supernatant absorbance at 405 nm with a Microplate ELISA Reader (Bio-Tek Instruments, VT, USA). Minimal haemolytic concentration (MHC) was defined as the minimal peptide concentration that produced 10% hemolysis. hRBCs in PBS (A_{blank}) or 0.1% Triton X-100 (A_{triton}) were used as the negative and positive controls, respectively. The haemolysis percentage was calculated according to the equation:

$$\% \text{ hemolysis} = 100 \times [(A_{sample} - A_{blank}) / (A_{triton} - A_{blank})].$$

2.6 Dye leakage

Calcein leakage from vesicles was determined by measuring the decrease in self-quenching. The fluorescence intensities of calcein released from large unilamellar vesicles (LUVs) composed of EYPE/EYPG (7:3, w/w) were monitored at 520 nm (excitation at 490 nm) on RF-5301PC spectrophotometer (Shimadzu, Japan) after 2 min incubation with peptide. The fluorescence intensity corresponding to 100% leakage was determined by addition of Triton X-100 to the sample (finally 0.1%, v/v).

2.7 Membrane depolarization

The membrane depolarization activity of individual peptides was determined by the membrane

potential-sensitive fluorescent dye, $diSC_3$-5. Briefly, *S. aureus* grown at 37°C with agitation to the mid-log phase (OD_{600} = 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 OD_{600} of 0.05 in similar buffer containing 0.1 M KCl. Subsequently, cells were incubated with 20 nM $diSC_3$-5 until stable reduction of fluorescence was achieved, implying incorporation of the dye into the bacterial membrane. An excitation wavelength of 622 nm and an emission wavelength of 660 nm were used to monitor depolarization. The membrane potential was fully dissipated by adding gramicidin D (finally 0.2 nM).

2.8 Measurement of nitric oxide (NO) release from LPS-induced RAW264.7 cells

RAW 264.7 cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2 and 95% air at 37 °C. Nitrite accumulation in culture media was used as an indicator of nitric oxide (NO) release. RAW264.7 macrophages were cultured overnight in 96-wells plate (5×10^5 cells/well). The medium was then removed followed by the addition to each well of fresh DMEM supplemented with 5% of bovine serum. The cells were stimulated with LPS (20 ng/ml) in the presence or absence of peptides. Cells that were stimulated with LPS alone, and untreated cells served as controls. After incubating for 24 h, the amount of NO in the supernatant was estimated from the accumulation of the stable NO metabolite nitrite with Griess reagent according to the manufacturers' instructions (1% sulfanilic acid, 0.1% N-1-Naphthylethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 540 nm.

3. Results and Discussion

3.1 Synthesis of 3 dimeric peptides with a disulphide bond

Each corresponding monomeric peptide (CKLKKLWKKLLK-NH_2, KLKKLWCKKLLK-NH_2 and KLKKLWKKLLKC-NH_2) of the 3 dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M and di-$K_6L_4W_1$-C) was purified to > 95% purity by reverse-phase high-performance liquid chromatography (RP-HPLC) on an analytical Vydac C_{18} column (Fig. 1). The 3 homo-dimeric peptides were prepared by oxidation of each of the corresponding monomeric peptides. Oxidation of each monomeric peptide was performed in 10% DMSO solution under oxygen atmosphere for 48 h at room temperature (peptide concentration, 1 mg/ml). The disulphide bond formation of each monomeric peptide while forming the 3 homo-dimeric peptides was monitored by RP-HPLC on an analytical C_{18} column (Fig. 1). The final purity of the 3 homo-dimeric peptides, as analyzed by analytical RP-HPLC, was > 95%. The correct molecular masses of the purified dimeric peptides were confirmed using matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan) (Table 1).

3.2 Antimicrobial and haemolytic activities

I examined the antimicrobial activity of the peptides against a representative set of bacterial strains, including 3 gram-negative bacteria (*Escherichia coli* [KCTC 1682], *Pseudomonas aeruginosa* [KCTC 1637] and *Salmonella typhimurium* [KCTC 1926]) and 3 gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC 1621]). The MIC values are shown in Table 2. All the peptides showed similar effective MIC values against all the bacterial strains. The monomeric peptide $K_6L_4W_1$ and the 2 dimeric peptides (di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C) had similar MIC values in the range of 6.25–25 μg/ml

against 6 different bacteria. In contrast, the antimicrobial activity of di-$K_6L_4W_1$-M was nearly 2-fold lower than that of the monomer $K_6L_4W_1$ (Table 2). Our results are in agreement with other studies that have shown that the dimerization in CAP11, PMAP-36, distinctin, and dicynthaurin does not affect the MIC values of the peptides [3, 13-15]. However, the dimeric peptide of halocidin showed a 4–20-fold increase in the permeabilization and antimicrobial activity compared to a monomeric peptide [16]. The disulphide-linked dimeric peptide of LLP1, derived from a lentivirus envelope protein, possesses much greater antimicrobial activity against *S. aureus* than monomeric LLP1 does [17]. In addition, disulphide-linked dimers of magainin 2 induce membrane permeabilization at lower concentrations than the monomeric form does [18]. These results suggest that the disulphide bond in various dimeric-AMPs seems only to partly contribute to their microbicidal effect. I next assessed the haemoytic effect of these peptides against mammalian cells by measuring their ability to cause lysis of human erythrocytes. The concentration-response curves for the haemolytic activity of the peptides are shown in Fig. 2-A. For a quantitative measure of the haemolytic activity of the peptides, I introduced the minimal haemolytic concentration (MHC) defined as the lowest peptide concentration that produces 10% haemolysis (Table 2). The MHC values for di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C were 140 μg/ml, and 47 μg/ml, respectively. However, these 2 peptides did not induce significant haemolysis at their MIC values observed against bacterial strains. $K_6L_4W_1$ and di-$K_6L_4W_1$-M did not cause haemolysis at the highest peptide concentration tested (400 μg/ml).

3.3 Therapeutic index

The therapeutic potential of the AMP drugs lies in the ability of the peptide to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. This property is defined by the concept of the therapeutic index (TI) as a measure of the relative safety of the drug [19, 20]. The TI of each peptide was calculated as the ratio of the MHC value to the geometric

mean (GM) of MICs against 6 selected microorganisms (Table 2). When haemolysis was significantly absent at the highest concentration tested (400 μM), 800 μM was used for the calculation of TI, since the test was carried out by 2-fold serial dilution. A high TI is, thus, an indication of 2 preferred characteristics of the peptide, namely, a high MHC (low haemolytic activity) and a low MIC (high antimicrobial activity) values. All the dimeric peptides had a lower TI than the monomeric peptide because of their increased haemolytic activity. Among the dimeric peptides, di-$K_6L_4W_1$-M had the highest TI. The order of TI for the peptides was $K_6L_4W_1$ > di-$K_6L_4W_1$-M > di-$K_6L_4W_1$-N > di-$K_6L_4W_1$-C.

3.4 Salt resistance

For effective use in clinical pharmacotherapy, AMPs need to remain active in the presence of physiological levels of salt (120–150 mm NaCl). To evaluate the effect of the position of a disulphide bond in homo-dimeric α-helical AMPs on their salt resistance, we examined the MICs of the peptides against 6 microorganisms and the bactericidal kinetics at a concentration of 2 × MIC toward *E. coli* in the presence or absence of 150 mM NaCl. When judged in terms of MIC value (Table 3), the monomeric peptide, $K_6L_4W_1$ displayed a 2–6-fold reduction in the antimicrobial activity in the presence of 150 mM NaCl, as compared to its activity in the absence of NaCl. Two dimeric peptides, di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C exhibited nearly unaltered antimicrobial activity against both gram-positive and gram-negative bacteria in the presence of 150 mM NaCl. In contrast, di-$K_6L_4W_1$-M did not show any antimicrobial activity even at the highest concentration tested (100 μg/ml). As shown in Fig. 2-B, the bactericidal kinetics of di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C were not suppressed at 150 mM NaCl. In contrast, the bactericidal kinetics of $K_6L_4W_1$ and di-$K_6L_4W_1$-M were significantly inhibited at high salt concentrations. Salt resistance of di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C may be due largely to their multimeric oligomerization at high concentration of NaCl. Lee et al. recently reported salt resistance of bactenecin homodimers is due

to increased multimeric oligomerization at high salt concentration [21]. These results indicate that a disulphide bond at the N- or C-terminus of the molecule, rather than a central position, is more effective with respect to antimicrobial activity when designing salt-resistant α-helical dimeric AMPs.

3.5 Mode of bactericidal action

To examine whether the antimicrobial activity of the peptides depend on their ability to permeate bacterial membranes, I measured their abilities to induce calcein leakage from negatively charged EYPE/EYPG (7:3 w/w) LUVs (bacterial cell membrane-mimicking environment). All of the peptides induced weak dye leakage of below 30% at 25μg/ml (Fig. 3-A). Next, to evaluate the effects of peptides on *S. aureus* cytoplasmic membranes, the membrane potential-sensitive fluorescent dye $diSC_3$-5 was used. This dye is distributed between the cells and the medium, depending on the cytoplasmic membrane potential, and self-quenches when concentrated inside bacterial cells. If the membrane is depolarized, this dye will be released into the medium, causing a measurable increase in fluorescence. All of the peptides caused significant membrane depolarization of above 90% at 12.5μg/ml (Fig. 3-B). These findings support the channel/pore formation model as the mechanism of bactericidal action of our designed peptides.

3.6 LPS-neutralizing activity

Sepsis is the major cause of mortality in the intensive care unit, accounting for 200,000 deaths every year in the United States alone [22]. Release of LPS from antibiotic-treated gram-negative bacteria can indeed enhance sepsis [12]. Therefore, an effective antimicrobial agent should not only exert antimicrobial activity but also have the ability to neutralize LPS. To investigate whether $K_6L_4W_1$ and its 3 dimeric peptides have LPS-neutralizing activity, as well as potent antimicrobial

activity, I assessed their ability to inhibit NO release in LPS-stimulated mouse macrophage RAW264.7 cells. $K_6L_4W_1$, di-$K_6L_4W_1$-N, and di-$K_6L_4W_1$-C significantly inhibited NO release in LPS-stimulated mouse macrophage RAW264.7 cells at a concentration of 20 μg/ml, 5 μg/ml and 5 μg/ml, respectively (Fig. 4). In particular, both di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C showed much greater inhibition of NO release compared to di-$K_6L_4W_1$-M (Fig. 4). This result indicates that a disulphide bond at the N- or C-terminus of the molecule is more effective than in a central position when designing α-helical dimeric AMPs with potent LPS-neutralizing activity. Taken together, our results will be useful for designing novel dimeric salt-resistant AMPs with potent antimicrobial and LPS-neutralizing activities.

Table 1. Amino acid sequences and calculated and observed molecular masses of the designed α-helical homo-dimeric model antimicrobial peptides

Peptides	Amino acid sequences	Molecular mass (Da)	
		Calculated	Observed
$K_6L_4W_1$	KLKKLWKKLLK-NH_2	1424.9	1424.8
di-$K_6L_4W_1$-N	(**C**KLKKLWKKLLK-NH_2)$_2$	3054.0	3053.3
di-$K_6L_4W_1$-M	(KLKKLW**C**KKLLK-NH_2)$_2$	3054.0	3052.9
di-$K_6L_4W_1$-C	(KLKKLWKKLLK**C**-NH_2)$_2$	3054.0	3053.3

Table 2. Antimicrobial and hemolytic activities and cell selectivity of the designed model antimicrobial peptides

Peptide	MIC[a] (μg/ml)						GM[b] (μg/ml)	MHC[c] (μg/ml)	Therapeutic Index[d] (MHC/GM)
	E. coli	*P. aeruginos*	*S. typhimuriu*	*B. subtilis*	*S. epidermidis*	*S. aureus*			
$K_6L_4W_1$	12.5	25	6.25	12.5	12.5	6.25	12.5	400 <	64
di-$K_6L_4W_1$-N	12.5	12.5	12.5	6.25	12.5	12.5	11.5	140	12.2
di-$K_6L_4W_1$-M	25	25	12.5	12.5	25	25	20.8	400 <	38.5
di-$K_6L_4W_1$-C	12.5	12.5	6.25	6.25	12.5	12.5	10.4	47	4.5

[a] MIC were determined in three independent experiments performed in triplicate.

[b] The geometric mean (GM) of the MIC values from all six bacterial strains in this table.

[c] The minimal peptide concentration (MHC) that produces 10% hemolysis. When no detectable hemolysis was observed at 400 μg/ml, I used a value of 800 μg/ml to calculate the therapeutic index.

[d] The ratio of the MHC (μg/ml) over the geometric mean (GM) of the MIC (μg/ml).

Table 3. Antimicrobial activity of the designed model antimicrobial peptides in the presence of 150 mM NaCl

Peptide	MIC (μg/ml)					
	E. coli	*P. aeruginosa*	*S. typhimurium*	*B. subtilis*	*S. epidermidis*	*S. aureus*
$K_6L_4W_1$	100	100	100	25	50	100
di-$K_6L_4W_1$-N	12.5	12.5	12.5	6.25	12.5	12.5
di-$K_6L_4W_1$-M	100 <	100 <	100 <	100 <	100 <	100 <
di-$K_6L_4W_1$-C	12.5	12.5	6.25	6.25	12.5	12.5

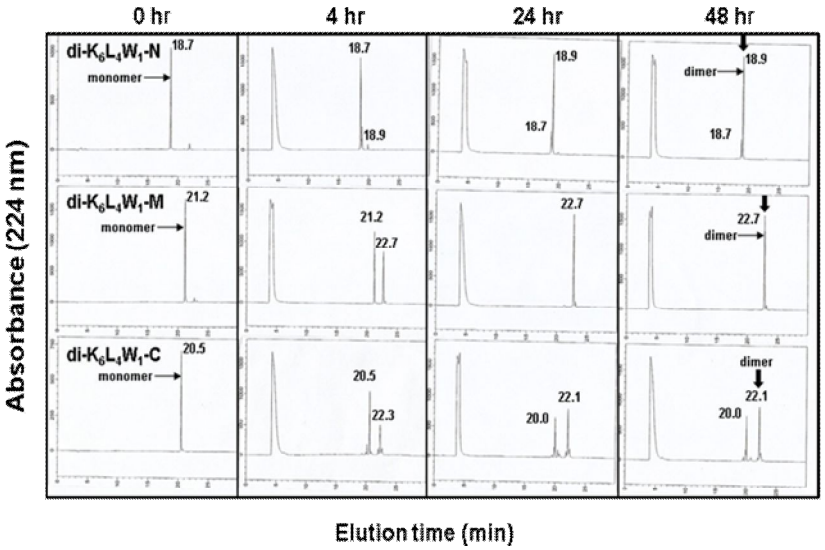

Fig. 1. The progression of a disulphide bond formation by each monomeric peptide forming the 3 homo-dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M, and di-$K_6L_4W_1$-C) as monitored by RP HPLC on an analytical C_{18} column (4.6 × 250 mm, 300Å, 5-μm particle size).

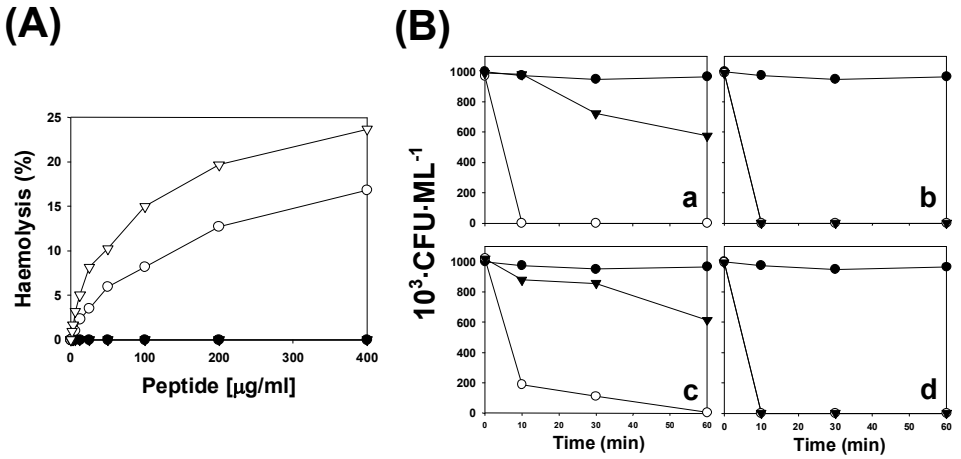

Fig. 2. Hemolytic activity and bactericidal kinetics of the peptides. (A) Concentration-response curves showing percent haemolysis of the peptides against human erythrocytes. Symbols: $K_6L_4W_1$ (●); di-$K_6L_4W_1$-N (○); di-$K_6L_4W_1$-M (▼); and di-$K_6L_4W_1$-C (▽). (B) Bactericidal kinetics of the peptides against *Escherichia coli.* Bacteria treated with the peptides (concentration, 2 × MIC) shown were diluted at the indicated times and then plated on Luria-Bertani agar. The CFUs were counted after 24 h of incubation at 37 °C. Symbols: Without peptide (●); 0 mM NaCl (○); 150 mM NaCl (▼). Peptides: a ($K_6L_4W_1$); b (di-$K_6L_4W_1$-N); c (di-$K_6L_4W_1$-M); and d (di-$K_6L_4W_1$-C).

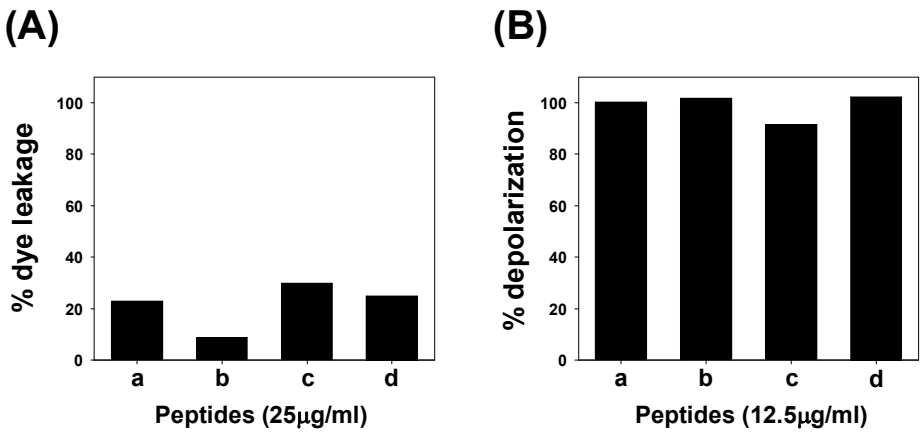

Fig. 3. (A) Percent dye leakage from negatively charged EYPE/EYPG (7:3, w/w) LUVs measured at 2 min after the addition the peptides. The concentration of EYPE/EYPG (7: 3, w/w) LUVs was 68 μM. (B) Percent membrane depolarization of *Staphylococcus aureus* (OD_{600} = 0.05) by the peptides using the membrane potential sensitive dye, $diSC_3$-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

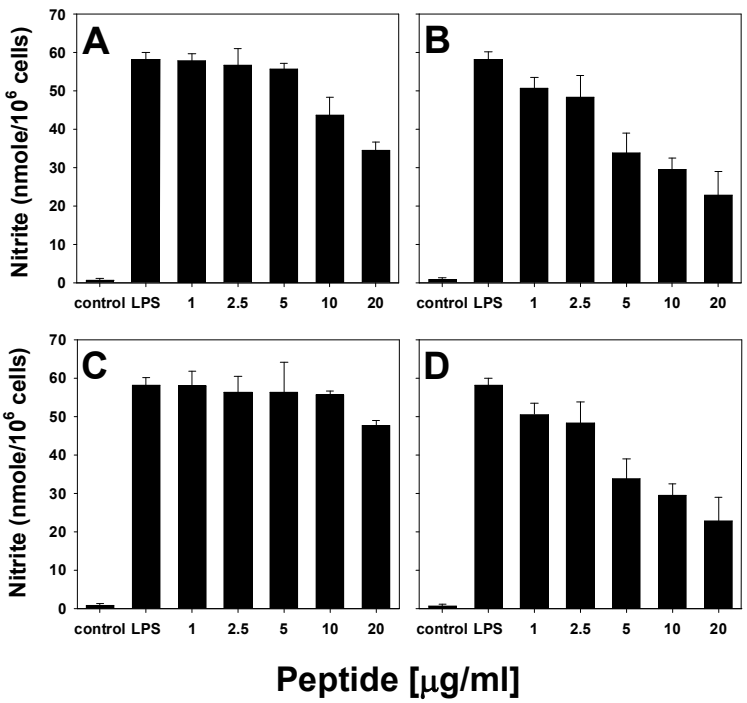

Fig. 4. Inhibitory activities of the peptides on LPS-stimulated nitric oxide (NO) production in RAW264.7 cells. RAW264.7 cells (5×10^5 cells/ml) were treated with 20 ng/ml LPS in the absence of or presence of various concentrations (1.0 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml and 20 μg/ml) of $K_6L_4W_1$ (A); di-$K_6L_4W_1$-N (B); di-$K_6L_4W_1$-M (C); and di-$K_6L_4W_1$-C (D). Error bars represent standard deviations of the mean determined from 3 independent experiments.

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PART Ⅲ

Mammalian cell toxicity and candidacidal mechanism of Arg- or Lys-containing Trp-rich model antimicrobial peptides and their D-enantiomeric peptides

1. Introduction

Antimicrobial peptides (AMPs) represent an important component of innate immunity and host defenses against infectious agents [4,11,41]. They have emerged as a potential new class of antimicrobial agents with new modes of actions [11,41]. There are AMPs which are rich in a certain specific amino acid such as Pro, Trp, Arg, or His [9]. The Trp residue has a high propensity to insert into membranes and to partition near the membrane–water interface [6,30]. The fact that Trp residues are located in the interfacial region of lipid bilayers has been well documented [13,14,30,40]. Due to these important features of Trp residue in peptide−membrane interaction, the mechanisms of action of several Trp-rich AMPs have been studied recently [6,10,12,20,27,30]. A well-studied example of a Trp-rich AMP is the 13-meric indolicidin (IN) isolated from the cytoplasmic granules of bovine neutrophils [32]. IN displays activity against a wide range of targets, including Gram-positive and Gram-negative bacteria, fungi, protozoa and enveloped virus HIV-1. However, in addition to its potent antibiotic potency, IN exhibits relatively strong hemolytic activity toward human erythrocytes and cytotoxic activity against mammalian cells. In our previous study, I synthesized a series of 11-mer cationic Trp-rich model AMPs, with different ratios of Lys and Leu residues, and with the structure XXWXXWXXWXX-NH_2 (X represents Leu or Lys), to develop novel Trp-rich model AMPs possessing higher cell selectivity (targeting bacterial cells but not mammalian cells), and that were shorter in length than IN [27]. Lys-containing Trp-rich model peptide ($K_6L_2W_3$: KLWKKWKKWLK-NH_2) showed approximately 4-fold higher cell specificity than did its counterpart Arg-containing Trp-rich model peptide ($R_6L_2W_3$: RLWRRWRRWLR-NH_2) [27]. This fact suggested that multiple Lys residues are more important than multiple Arg residues in the design of AMPs with improved cell specificity. Furthermore, $K_6L_2W_3$ and $R_6L_2W_3$ and their D-enantiomeric peptides composed of D-amino acids ($K_6L_2W_3$-D and $R_6L_2W_3$-D) penetrated the cell membrane and accumulated in the cytoplasm of *E. coli.* In addition, I confirmed that these model peptides bind strongly to DNA by gel retardation assay [27]. These results

indicated that a possible mechanism of antimicrobial action of these Trp-rich model peptides may be related to the inhibition of intracellular functions via interference with DNA/RNA synthesis.

Candida albicans is an opportunistic fungal pathogen that is a leading cause for mucosal and systemic candidiasis in people [5,21]. The cytotoxicity of antifungal agents and the emergence of strains resistant to currently used antifungal drugs, such as fluconazole and amphotericin B [29] have renewed interest in the development of novel approaches to the design of peptide-based antifungal agents that are less harmful for host cells and that possess a low tendency to select for resistant strains. A number of antimicrobial peptides, including histatin 5, lactoferrin-derived peptides, and cathelicidin peptides have shown potent fungicidal activities against *C. albicans* [3,19,37].

In this study, I found that $K_6L_2W_3$ and $R_6L_2W_3$ and their D-enantiomeric peptides, $K_6L_2W_3$-D and $R_6L_2W_3$-D have a potent antifungal activity against *Candida albicans* with MIC ranging between 32 μM to 64 μM. To evaluate the cytotoxicity of these peptides to mammalian cells, I examined the hemolytic activity against human erythrocytes and cytotoxic activity against three different types of mammalian cells, mouse fibroblastic NIH-3T3 cells, human cervical carcinoma HeLa cells and human keratinocyte HaCaT cells.

To gain insight into the molecular basis for the specificity of the peptides between the fungal and mammalian cells, I investigated the interaction of the peptides with model liposome systems which mimic the outer membrane of fungal or mammalian cells. Additionally, the leakage of calcein from PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) liposomes which mimic the outer leaflet of the plasma membrane of *C. albicans* was performed to assess the question, whether anti-*Candida* activity of these model peptides might depend on peptide ability to permeate fungal membranes. To identify the major target site of candidacidal action of these peptides, the association of the peptides with *C. albicans* was visualized using confocal laser-scanning microscopy.

2. Materials and methods

2.1. Materials

Rink amide 4-methylbenzhydrylamine (MBHA) resin, fluoren-9-yl-methoxycarbonyl (Fmoc)-amino acids and other reagents for the peptide synthesis were purchased from Calbiochem-Novabiochem (La Jolla, CA). Posphatidylcholine (PC, from egg yolk), phosphatidylglycerol (PG, from egg yolk), phosphatidylethanolamine (PE, from egg yolk), phosphatidylinositol (PI, from soybean), cholesterol, ergosterol, acrylamide and calcein were supplied from Sigma Chemical Co (St. Louis, MO). All other reagents were of analytical grade. The buffers were prepared in double glass-distilled water.

2.2. Peptide synthesis

The peptides and FITC-labeled peptides were prepared by the standard Fmoc-based solid-phase synthesis technique on a solid support of Rink amide MBHA resin. DCC (dicyclohexylcarbodiimide) and HOBt (N-hydroxybenzotriazole) were used as coupling reagent, and ten-fold excess Fmoc-amino acids were added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/water/thioanisole/phenol/ethanedithiol/triisopropylsilane (81.5:5:5:5:2.5:1, v/v/v/v/v/v) for 2 h at room temperature, the crude peptides were repeatedly extracted with diethyl ether and purified by reverse phase HPLC on a preparative Vydac C_{18} column (15 μm, 20 × 250 mm) using an appropriate 0–90% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. The final purity of the peptides (>98%) was assessed by reverse phase HPLC on an analytical Vydac C_{18} column (4.6 mm × 250 mm, 300 Å, 5-μm particle size). The molecular mass of the purified peptides was determined by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-

flight mass spectrometry) (Shimadzu, Japan) (data not shown).

2.3. RP-HPLC analysis of peptides

Each peptide was subjected to a RP-HPLC using an analytical Vydac C_{18} column (4.6 mm × 250 mm). The peptides were eluted using a linear gradient of 5–45% acetonitrile in 0.1% trifluoroacetic acid at flow rate of 1 ml/min for 60 min. The RP-HPLC retention time of each peptide was determined when the peak was at its maximum height.

2.4. Anti-Candida Activity

The antifungal activity of the peptides against *Candida albicans* [KCTC 7965] was determined by the broth microdilution assay. Briefly, single colonies of *C. albicans* were inoculated into the culture media (YPD broth) (2% dextrose, 1% peptone, 0.5% yeast extract) and cultured overnight at 28°C. An aliquot of these cultures were transferred to 10 ml fresh culture medium and incubated for an additional 3–5 h at 28°C to obtain mid-logarithmic phase organisms. A 2-fold dilution series of peptides in PBS was prepared. A set of serial dilutions (100 μl) were added to 100 μl of 1×10^5 CFU/ml in 96-well microtiter plates (Falcon) and then incubated at 28°C for 16 h. The lowest concentration of peptide that completely inhibited growth of the organisms was defined as the minimal inhibitory concentration (MIC). The MICs were the average of triplicate measurements in three independent assays. *C. albicans* [KCTC 7965] was procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

2.5. Hemolytic activity

Hemolytic activity of the peptides was tested against human red blood cells (h-RBCs). Fresh h-

RBCs were washed three times with phosphate-buffered saline (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-RBCs 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.

2.6. Cytotoxicity against mammalian cells

Cytotoxicity of the peptides against mammalian cells was determined by the MTT assay [27,31]. Mouse fibroblastic NIH-3T3 cells, human cervical carcinoma HeLa cells and human keratinocyte HaCaT cells were cultured in DMEM with 10% FBS. The cells were maintained under 5% CO_2 at 37 °C. The cells were seeded on 96-well microplates at a density of 1×10^5 cells/well in 150 μl of DMEM containing 20% FBS. The plates were then incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Twenty microlilters of peptide solution (serial 2-fold dilutions in DMEM) was added, and the plates were incubated for a further 2 days. Wells containing cells without peptides served as controls. Subsequently, 20 μl of an MTT solution (5 mg/ml) was added to each well, and the plates were incubated for a further 4 h at 37 °C. The precipitated MTT formazan was dissolved in 40 μl of 20% (w/v) SDS containing 0.01 M HCl overnight. The absorbance at 570 nm was measured using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA). Percent cell survival was expressed as a percent ratio of A_{570} of cells treated with peptide over cells only.

2.7. Preparation of small unilamellar vesicles (SUVs)

SUVs (small unilamellar vesicles) were prepared by sonication as described earlier [33]. Briefly, dry lipids were dissolved in chloroform in a small glass vessel. Solvents were removed by rotary evaporation to form a thin film on the wall of a glass vessel and lyophilized overnight. Dried thin films were resuspended in Tris–HCl buffer by vortex mixing. Lipid dispersions were sonicated on ice water for 10–20 min with a titanium-tip ultrasonicator until the solution became transparent. Two different compositions of lipid films were prepared: PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) and PC/cholesterol (10:1, w/w), which mimics the outer leaflet of the plasma membrane of *C. albicans* [1,18] and human red blood cells (h-RBCs) [36], respectively.

2.8. Quenching of Trp emission by acrylamide

For fluorescence quenching experiment, acrylamide was used as the quencher and the fluorescence measurements were taken with a model RF-5301PC spectrophotometer (Shimadzu, Japan). To reduce absorbance by acrylamide, excitation of Trp at 295 nm instead of 280 nm was used [7,42]. Aliquots of the 3.0 M solution of this water-soluble quencher were added to the peptide in presence of 0.6 mM PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) SUVs at a peptide/lipid molar ratio of 1:200. The effect of acrylamide on the fluorescence of each peptide was analyzed by a Stern–Volmer equation: $F_0/F = 1 + Ksv\ [Q]$, where F_0 and F represent the fluorescence intensities in the absence and the presence of acrylamide, respectively, and Ksv is the Stern–Volmer quenching constant and [Q] is the concentration of acrylamide.

2.9. Dye leakage

Calcein-entrapped LUVs (large unilamellar vesicles) composed of PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) were prepared by vortexing the dried lipid in dye buffer solution (70 mM calcein, 10mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was subjected to 10 frozen-thaw cycles in liquid nitrogen and extruded 21 times through polycarbonate filters (two

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. The concentration of calcein-entrapped LUVs was determined in triplicate by phosphorus analysis [2]. Calcein leakage from LUVs was monitored at room temperature by measuring fluorescence intensity at an excitation wavelength of 490 nm and emission wavelength of 520 nm on a model RF-5301PC spectrophotometer. Complete dye release was obtained by using 0.1% Triton X-100.

2.10. Confocal laser-scanning microscopy

C. albicans cells were grown to the mid-logarithmic phase. The cells (1×10^6 CFU/ml) in 10 mM PBS, pH 7.4 were incubated with FITC-labeled peptides (10 μg/ml) at 28 °C for 30 min. Next, the cells were washed with PBS and immobilized on a glass slide. FITC-labeled peptides were observed with an Olympus IX 70 confocal laser-scanning microscope (Japan). Fluorescent images were obtained with a 488-nm band-pass filter for FITC excitation.

3. Results

3.1. Anti-Candida and hemolytic activities

Lys-containing peptides ($K_6L_2W_3$ and $K_6L_2W_3$-D) and Arg-containing counterpart peptides ($R_6L_2W_3$ and $R_6L_2W_3$-D) exhibited a similar anti-*Candida* activity with MIC ranging between 32 μM to 64 μM (Table 1). In the hemolytic activity, IN displayed approximately 60% hemolysis at the high peptide concentration of 400μM, but all of model peptides exhibited below 15% hemolysis (Figure 1). Interestingly, Arg-containing peptides exerted slightly higher hemolytic activity than Lys-containing peptides (Figure 1).

3.2. Cytotoxicity against mammalian cells

The cytotoxicity of these model peptides against three different types of mammalian cells, NIH-3T3, HeLa and HaCaT cells was determined by the MTT assay. As shown in Figure 2, Arg-containing peptides displayed much higher cytotoxicity as compared to Lys-containing peptides.

3.3. Hydrophobicty

Hydrophobicity of the peptides was determined by measuring the RP-HPLC retention times as previously reported [15]. As shown in Figure 3, Lys-containing peptides were eluted somewhat earlier (1.3~1.5 min) from analytical RP-HPLC column than Arg-containing counterparts, indicating Arg-containing peptides is slightly more hydrophobic than Lys-containing counterparts.

3.4. Quenching of Trp emission by acrylamide

To investigate the relative extent of peptide burial in liposomes and the lipid specificity of the peptide–liposome interaction, I examined the effect of acrylamide, a water soluble neutral quencher of tryptophan fluorescence. Acrylamide is useful as a quenching agent because it does not interact with the head group of negatively charged phospholipids. The Stern–Volmer plots for the quenching of tryptophan by acrylamide in Tris-HCl buffer or in the presence of PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) SUVs or PC/cholesterol (10:1, w/w) SUVs are depicted in Figure 4. The tryptophan fluorescence for all model peptides was decreased in a concentration-dependent manner by the addition of acrylamide. In negatively charged PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) liposomes, all model peptides showed similar slopes and Ksv. In contrast, in zwitterionic PC/cholesterol (10:1, w/w) liposomes, Lys-containing peptides exerted slightly higher slope and larger Ksv than Arg-containing peptides, suggesting that the Trp residue of Lys-containing peptides is less anchored within the hydrophobic core of the zwitterionic phospholipids as compared to Arg-containing peptides (Figure 4 and Table 2).

3.5. Dye leakage

To examine whether anti-*Candida* activity of these model peptides might depend on peptide ability to permeate fungal membranes, I measured the influence of peptides on calcein leakage from PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) LUVs (Figure 5). Lys-containing peptides induced much weaker dye leakage at a peptide:lipid molar ratio of 1:32, than Arg-containing peptides.

3.6. Confocal laser-scanning microscopy

To monitor the site of action of the peptides in *C. albicans*, FITC-labeled Lys- or Arg-containing peptide with *C. albicans*, and their localization within *C. albicans* was visualized under confocal laser-scanning microscopy. The FITC-labeled peptides purified using C_{18} RP- HPLC were about

95% homogeneous by MALDI-TOF MS (data not shown). At a concentration of 10 μg/ml, FITC-labeled Lys-containing peptides penetrated the cell membrane and accumulated in the cytoplasm of the cell (Figure 6). On the other hand, FITC-labeled Arg-containing peptides did not penetrate the cell membrane but associated with the cell membranes (Figure 6). Thus it seems that the target site of Arg-containing peptides and Lys-containing peptides is the membrane and the cytoplasm of *C. albicans,* respectively.

Table 1. Amino acid sequences, RP-HPLC retention times and anti-*Candida* activity of the peptides

Peptides	Amino acid sequences	RP-HPLC retention times (min)	*Candida albicans* [KCTC 7965] MIC (μM)
$K_6L_2W_3$	KLWKKWKKWLK-NH_2	25.86	32
$K_6L_2W_3$-D	klwkkwkkwlk-NH_2	25.89	32
$R_6L_2W_3$	RLWRRWRRWLR-NH_2	27.21	64
$R_6L_2W_3$-D	rlwrrwrrwlr-NH_2	27.32	32

Small letters represent D-amino acids. Retention time (RT) was measured using a C_{18} reverse-phase analytical column (5 μm; 4.6×250 mm; Vydac). Peptides were eluted for 60 min, using a linear gradient of 5% to 45% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid.

Table 2. Stern–Volmer quenching constant (Ksv) in Tris-HCl buffer or in the presence of liposomes of the peptides

Peptides	Ksv (M^{-1})		
	Tris-HCl buffer (pH 7.2)	PC/PE/PI/ergosterol (5/2.5/2.5/1, w/w/w/w)	PC/cholesterol (10:1, w/w)
$K_6L_2W_3$	7.81	2.20	5.74
$K_6L_2W_3$-D	7.81	2.20	5.55
$R_6L_2W_3$	6.96	2.19	4.74
$R_6L_2W_3$-D	6.65	1.96	4.14

Assays were carried out in Tris-HCl buffer or in the presence of PC/PE/PI/ergosterol (5/2.5/2.5/1, w/w/w/w) or PC/cholesterol (10:1, w/w) liposomes at a lipid／peptide molar ratio of 100:1.

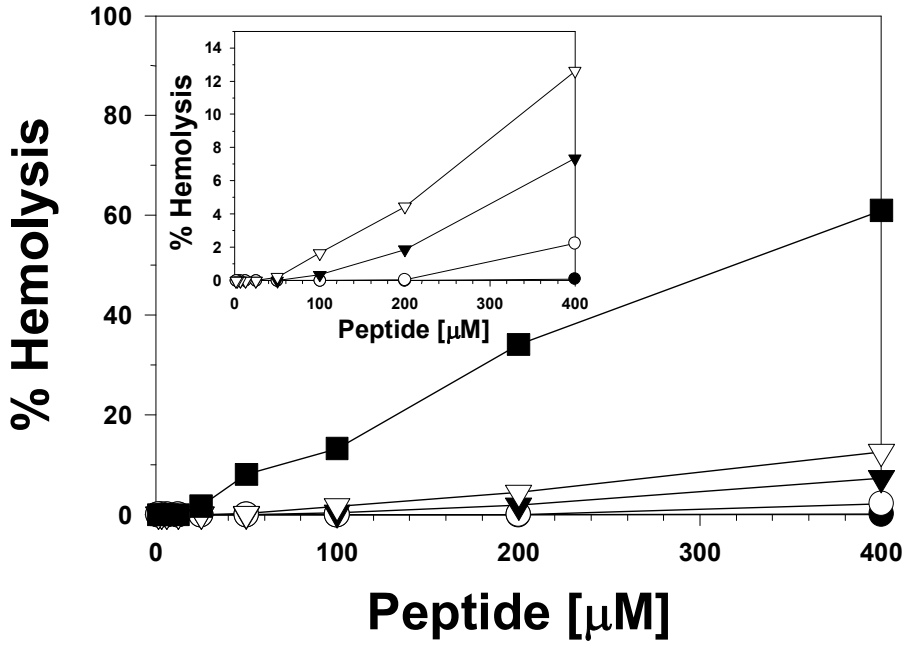

Fig. 1. Concentration-response curves of percent hemolysis of designed model peptides towards human erythrocytes. Peptides are indicated as follows: $K_6L_2W_3$ (●); $K_6L_2W_3$-D (○); $R_6L_2W_3$ (▼); $R_6L_2W_3$-D (▽); Indolicidin (■).

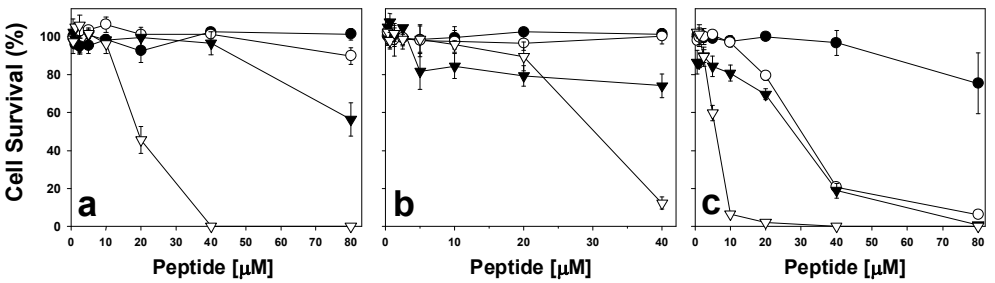

Fig. 2. Cytotoxicity of the peptides against mouse fibroblastic NIH-3T3 cells (a), human cervical carcinoma HeLa cells (b) and human keratinocyte HaCaT cells (c). Peptides are indicated as follows: $K_6L_2W_3$ (●); $K_6L_2W_3$-D (○); $R_6L_2W_3$ (▼); $R_6L_2W_3$-D (▽).

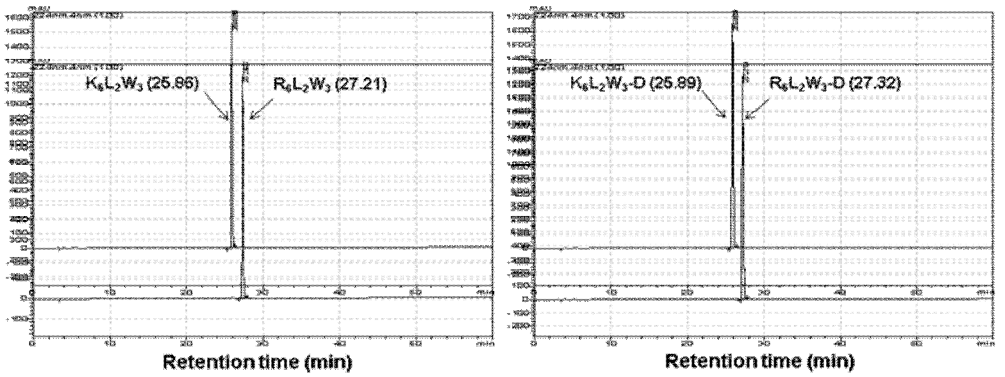

Fig. 3. Analytical reverse phase-high performance liquid chromatography (RP-HPLC) profile of the peptides. Retention time (RT) was measured using a C_{18} reverse-phase analytical column (5 μm; 4.6 mm × 250 mm; Vydac). Peptides were eluted for 60 min using a linear gradient of 5% to 45% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid. The absorbance was measured at 224 nm.

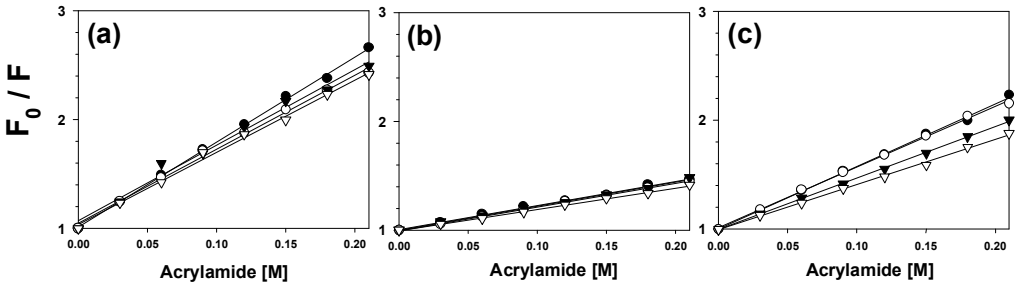

Fig. 4. Stern–Volmer plots for the quenching of Trp fluorescence of the peptides by an aqueous quencher, acrylamide, in Tris-HCl buffer (pH 7.2) (a) in the presence of PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) SUVs (b) and PC/cholesterol (10:1, w/w) SUVs (c). Peptides are indicated as follows: $K_6L_2W_3$ (●); $K_6L_2W_3$-D (○); $R_6L_2W_3$ (▼); $R_6L_2W_3$-D (▽).

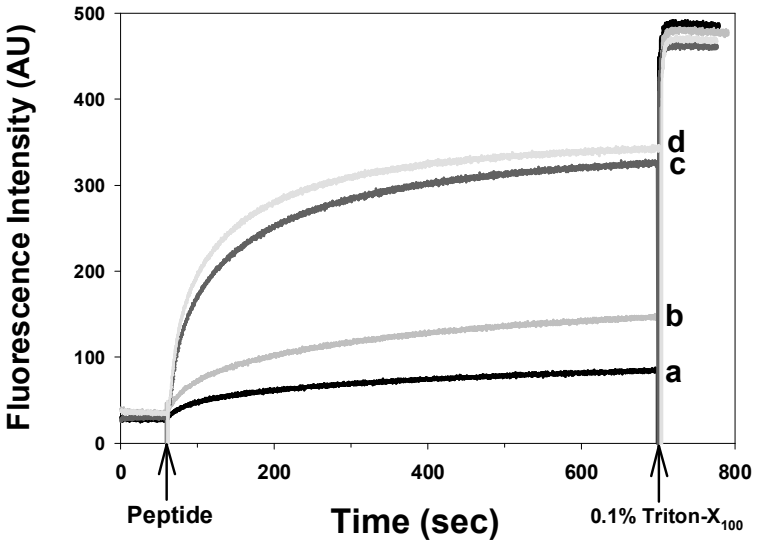

Fig. 5. Peptide-induced dye leakage from PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) LUVs. The concentration of the peptides was 8 μM. The concentration of the liposome was 256 μM. Peptides are indicated as follows: $K_6L_2W_3$ (a); $K_6L_2W_3$-D (b); $R_6L_2W_3$ (c); $R_6L_2W_3$-D (d).

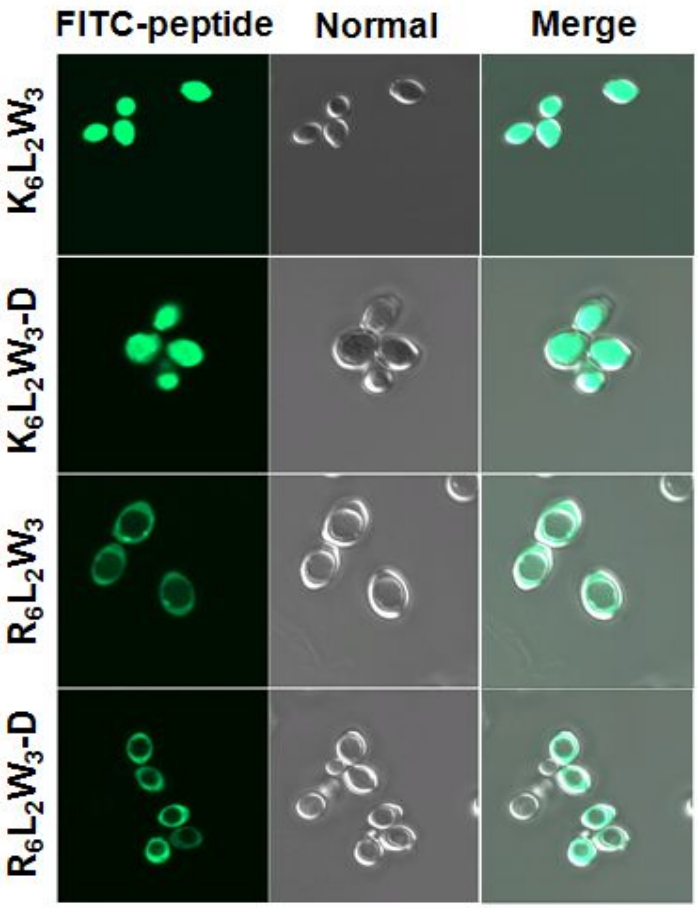

Fig. 6. Confocal laser-scanning microscopy of *C. albicans* treated with FITC-labeled peptides. The cells were incubated with 10 μg/ml of FITC-labeled peptides at 28°C for 30 min. Panels on the left, middle and right represent laser-scanning images of FITC-labeled peptides of *C. albicans*, normal image and merged image, respectively.

4. Discussion

Although antimicrobial peptides (AMPs) show great structural diversity, they are cationic, because they have multiple Arg and/or Lys residues [8,9,16,35]. These cationic amino acids are thought to be very important for binding to negatively charged surfaces of microbial lipid membranes [8,9,16,35]. In this study, Arg-containing peptides ($K_6L_2W_3$ and $K_6L_2W_3$-D) exerted slightly higher hemolytic activity than Lys-containing peptides ($R_6L_2W_3$ and $R_6L_2W_3$-D). Arg-containing peptides displayed much more cytotoxicity against three different mammalian cells than Lys-containing peptides. Net positive charge, helicity, hydrophobic moment, angle subtended by charged residues and hydrophobicity have been suggested as major parameters influencing the biological activity of α-helical AMPs [22,28,38,39]. Among these, the hydrophobicity of AMPs has been recognized as important factor to increase hemolytic activity and mammalian cell cytotoxicity [18,24,34,35]. Therefore, I examined the hydrophobicity of these model peptides using RP-HPLC retention time [15]. I found that Arg-containing peptides are slightly more hydrophobic than Lys-containing counterparts. These results suggested that a little difference in hydrophobicity of these peptides affect their hemolytic activity and mammalian cell toxicity. Interestingly, $K_6L_2W_3$ and its D-enantiomeric peptide, $K_6L_2W_3$-D almost similar cytotoxicity against three different types of mammalian cells, whereas $R_6L_2W_3$-D showed much higher cytotoxicity than $R_6L_2W_3$.

Many AMPs form either α-helical or β-sheet structures that exhibit a strong amphipathic nature. After the initial electrostatic attraction to an anionic microbial surface, these cationic amphipathic peptides can spontaneously insert into cell membranes and form pores/channels, causing lysis of the cell membrane (*i.e.,* membrane-targeting AMPs) [24,25,34]. In contrast to pore-forming AMPs, intracellular-targeting AMPs would translocate across the plasma membrane, distorting its structure in a transient, non-lytic manner, and once inside the cell would recognize intracellular targets such as DNA/RNA (buforin-II) [26], DnaK (drosocin and phyrrhocorrycin) [23], and mitochondria (histatin 5) [17,19].

To examine whether anti-*Candida* activity of these model peptides might depend on peptide ability to permeate the cell membranes of *C. albicans*, I measured the ability of peptides to facilitate fluorescent marker escape from PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w/w/w) liposomes that mimics the outer membrane of *C. albicans*. As the results, Lys-containing peptides induced much less dye leakage than Arg-containing peptides, suggesting the ultimate target site of Lys-containing peptides may be not the membrane but the cytoplasm of *C. albicans*.

Furthermore, to visualize the association of these model peptides with *C. albicans*, I incubated *C. albicans* with FITC-labeled peptides and then visualized their cellular localization by confocal laser-scanning microscopy. FITC-labeled Lys-containing peptides penetrated the cell wall and cell membrane and accumulated inside the cells, whereas FITC-labeled Arg-containing peptides did not penetrate but associated with the membranes. This finding indicates that the ultimate targeting site of action of Arg-containing peptides and Lys-containing peptides may be the membrane and the cytoplasm of *C. albicans,* respectively.

Collectively, our results suggested that the candidacidal activity of Arg-containing peptides and Lys-containing peptides may be due to the formation the transmembrane pore/channel or disrupting of the fungal cytoplasmic membranes and the inhibition of intracellular functions, respectively. Finally, $K_6L_2W_3$, $K_6L_2W_3$-D and $R_6L_2W_3$ with potent anti-*Candida* activity but no or less hemolytic activity and mammalian cell cytotoxicity may be potentially a useful lead compound for the development of novel antifungal agents.

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ABSTRACT

Cell Selectivity, Mechanism of Action and Anti-inflammatory Activity of Cathelicidin-derived and Model Antimicrobial Peptides

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PART Ⅰ

Human cathelicidin antimicrobial peptide (AMP), LL-37 has been shown to have the potential to inhibit lipopolysaccharide (LPS)-induced cellular cytokine release, as well as its direct antimicrobial function. In order to develop novel AMPs with shorter in length, improved prokaryotic selectivity and retained anti-inflammatory activity compared to natural LL-37, a series of amino acid-substituted analogs based on IG-19 (residues 13-31 of LL-37) were synthesized. There was a significant linear correlation between the hydrophobicity of IG-19 and its analogs and their prokaryotic selectivity or anti-inflammatory activity. Among IG-19 analogs, the analog a4 showed the highest prokaryotic selectivity (a4 had therapeutic index enhanced 7.1-fold over that of LL-37), but much lower anti-inflammatory compared to LL-37. The analogs, a5, a6, a7, a8 and a9 with higher hydrophobicity had strong anti-inflammatory activity comparable to that of LL-37, but poor prokaryotic selectivity (TI values: 0.1~0.5). These results indicated that the appropriate hydrophobicity of the peptides to exert good prokaryotic selectivity and anti-inflammatory activity

is of great importance.

In addition, to retain the prokaryotic selectivity and increase anti-inflammatory activity of the analog a4, I synthesized Trp-substituted analogs (a4-W1 and a4-W2) which Phe^{15} or Phe^{5} of a4 are replaced by Trp, respectively. Both a4-W1 and a4-W2 showed the same prokaryotic selectivity, but a4-W1 displayed much higher anti-inflammatory activity, compared to a4-W2. These results suggested that the effective site for the Trp-substitution in designing novel AMPs having higher anti-inflammatory activity without a significant reduction in prokaryotic selectivity is the amphipathic interface between the hydrophilic ending side and the hydrophobic starting side in the helical wheel projection and other important parameters of AMPs may be involved in their anti-inflammatory activity, as well as their net positive charge and hydrophobicity.

Furthermore, to provide the stability to proteolytic digestion of designed peptides, I synthesized the diastereomeric peptides (a4-W1-D and a4-W2-D) with D-amino acid substitution at positions 3, 7, 10, 13 and 17 of a4-W1 and a4-W2, respectively and their D-enantiomeric peptides (a4-W1-E and a4-W2-E) composed D-amino acids. After tryptic digestion, these diastereomeric and D-enantiomeric peptides preserved their antimicrobial activity almost completely. D-diastereomeric peptides exhibited the best prokaryotic selectivity and protease resistance, but much low anti-inflammatory activity. D-enantiomeric peptides showed prokaryotic selectivity, anti-inflammatory activity and protease resistance.

In conclusion, a4-W1, a4-W1-E and a4-W2-E with more improved prokaryotic selectivity and retained anti-inflammatory activity compared to parental LL-37 could serve as the templates for the development of antimicrobial agents for the treatment of sepsis, as well as microbial infection.

PART Ⅱ

To investigate the effects of the position of a disulphide bond on the salt resistance and

lipopolysaccharide (LPS)-neutralizing activity of α-helical homo-dimeric antimicrobial peptides (AMPs), I synthesized an α-helical model peptide ($K_6L_4W_1$) and its homo-dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M, and di- $K_6L_4W_1$-C) with a disulphide bond at the N-terminus, the central position, and the C-terminus of the molecules, respectively. Unlike $K_6L_4W_1$ and di-$K_6L_4W_1$-M, the antimicrobial activity of di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C was unaffected by 150 mM NaCl. Both di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C caused much greater inhibitory effect on nitric oxide (NO) release in LPS-induced mouse macrophage RAW264.7 cells, compared to di-$K_6L_4W_1$-M. Taken together, our results indicate that the presence of a disulphide bond at N- or C-terminus of the molecule, rather than at the central position, is more effective when designing salt-resistant α-helical homo-dimeric AMPs with potent antimicrobial and LPS-neutralizing activities.

PART Ⅲ

In this study, I investigated the mammalian cell toxicity and candidacidal mechanism of Arg- or Lys-containing Trp-rich model antimicrobial peptides ($K_6L_2W_3$ and $R_6L_2W_3$) and their D-enantiomeric peptides ($K_6L_2W_3$-D and $R_6L_2W_3$-D). Arg-containing peptides were more toxic to human erythrocytes and mammalian cells as compared to Lys-containing peptides. Arg-containing peptides are slightly more hydrophobic than Lys-containing counterparts, as judged from their reverse phase-high performance liquid chromatography (RP-HPLC) retention time. These results suggested that a little difference in hydrophobicity of these peptides affect their hemolytic activity and mammalian cell toxicity. Interestingly, $K_6L_2W_3$ and $K_6L_2W_3$-D almost similar mammalian cell cytotoxicity, whereas $R_6L_2W_3$-D showed much higher cytotoxicity as compared to $R_6L_2W_3$. A low ability to facilitate fluorescent marker escape from *C. albicans* membrane-mimicking vesicles suggested that the major target site of Lys-containing peptides may be not the cell membrane but the cytoplasm of *C. albicans*. Confocal laser-scanning microscopy revealed that FITC-labeled Lys-containing peptides penetrated the cell wall and cell membrane and accumulated inside the cells,

whereas FITC-labeled Arg-containing peptides did not penetrate but associated with the membranes. Collectively, our results suggested that the ultimate target site of action of Arg-containing peptides and Lys-containing peptides may be the membrane and the cytoplasm of *C. albicans,* respectively.

Curriculum Vitae

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Educational and Personal Experiences

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2006~2008: M.S., Department of Medicine, Chosun University, Kwangju, Korea

M.S. Thesis: Studies on Antimicrobial and Anti-inflammatory Activities of Trp-rich Antimicrobial Peptide Indolicidin and Its Analogues

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Scientific Activities

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21. Antimicrobial and LPS-neutralizing activities of bovine myeloid antimicrobial peptide-18 (BMAP-18) analogs.

Yong Hai Nan, Eun Kyu Lee and Song Yub Shin. 2011 KSBMB Annual Meeting, COEX Center, Seoul, Korea, May16-18, 2011

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무엇보다도 든든한 후원자인 너무나 사랑하고 보고 싶은 우리 가족, 아버지, 어머니, 누나 그리고 나의 동력이자 활력소인 여자친구 류련금에게 이 영광을 같이 하고 싶습니다. 그들이 있었기에 이렇게 유학생활을 이렇게 잘 마칠 수 있게 되였습니다. 앞으로 더욱 효도하고 사랑해주는 아들과 남편으로 행복하게 해드리겠습니다.

그 외에도 많은 분들의 도움을 받았지만 말보다 행동으로 그들에게 보답하겠습니다.