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Prokaryotic Selectivity, Anti-endotoxic Activity and Protease Stability of Diastereomeric and Enantiomeric Analogs of Human Antimicrobial Peptide LL-37

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LL-37 is the only antimicrobial peptide (AMP) of the human cathelicidin family. In addition to potent antimicrobial activity, LL-37 is known to have the potential to inhibit lipolysaccharide (LPS)-induced endotoxic effects. To provide the stability to proteolytic digestion and increase prokaryotic selectivity and/or anti-endotoxic activity of two Lys/Trp-substituted 19-meric antimicrobial peptides (a4-W1 and a4-W2) designed from IG-19 (residues 13-31 of LL-37), we 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 the enantiomeric peptides (a4-W1-E and a4-W2-E) composed D-amino acids. The diastereomeric peptides exhibited the best prokaryotic selectivity and effective protease stability, but no or less anti-endotoxic activity. In contrast, the enantiomeric peptides had not only prokaryotic selectivity and anti-endotoxic activity but also protease stability. Our results suggest that the hydrophobicity and α-helicity of the peptide is important for anti-endotoxic activity. In particular, the enantiomeric peptides showed potent anti-endotoxic and LPS-neutralizing activities comparable to that of LL-37. Taken together, both a4-W1-E and a4-W2-E holds promise as a template for the development of peptide antibiotics for the treatment of endotoxic shock and sepsis.

Key Words : Diastereomeric LL-37 analogs, Enantiomeric LL-37 analogs, Prokaryotic selectivity, Anti-endotoxic activity, Protease stability

Introduction

The cathelicidins are a group of structurally diverse antimicrobial peptides (AMPs) found in several mammalian species.[1] LL-37 is the only AMP of the human cathelicidin family.[2-4] It is produced from the C-terminal end of a precursor cathelicidin protein hCAP-18 by proteolysis.[5,6] LL-37 has been shown to be secreted by a large number of cell types, including neutrophils, keratinocytes and epithelial cells.[7] 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. LL-37 has a broad-spectrum antimicrobial activity against a wide range of gram-positive and gram-negative bacteria *in vitro*.[8]

Lipolysaccharide (LPS; known also as endotoxin) is an integral structural component the outer membrane of gram-negative bacteria, and is thought to be a major mediator of sepsis and septic shock caused by bacterial infection. In addition to its potent antimicrobial activity, LL-37 has been known to inhibit the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) and other inflammatory mediators such as nitric oxide (NO) in response to LPS in immune cells.[9-12] Therefore, an amalgamated property of antimicrobial and anti-endotoxic activities makes LL-37 an attractive drug candidate for treatment of endotoxin shock and sepsis.

In the previous study, we developed two Lys/Trp-substituted 19-meric analogs (a4-W1 and a4-W2) based on IG-19 (residues 13-31 of LL-37) with prokaryotic selectivity (means having killing activity selectively against prokaryotic cells without mammalian cell toxicity) and anti-endotoxic activity.[13] IG-19 is known as the α-helical region and the active domain for the modulation of TLR (Toll-like receptor) responses of LL-37.[11,14] As shown in their α-helical wheel projections, these two peptides (a4-W1 and a4-W2) was designed to have a nearly perfect amphipathic α-helical structure with hydrophilic and hydrophobic faces by introducing four lysines (E^4, Q^{10}, D^{14}, $N^{18}\rightarrow K$) and one tryptophan (F^5 or $F^{15}\rightarrow W$).[13] Both a4-W1 and a4-W2 have the same net positive charge of +11, and a nearly identical hydrophobicity and percent α-helicity. These two peptides show the same prokaryotic selectivity (therapeutic index = 2.8), but a4-W2 displayed much higher anti-endotoxic activity compared to a4-W1.[13]

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.[15,16] In the present study, to provide the stability to proteolytic digestion and increase prokaryotic selectivity and/or anti-endotoxic activity of a4-W1 and a4-W2, we 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 enantiomeric peptides (a4-W1-E

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and a4-W2-E) composed D-amino acids. 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. Anti-endotoxic activity of the peptides was evaluated by examining inhibition of NO production and TNF-α secretion in LPS-stimulated RAW264.7 cells, a mouse macrophage cell line. LPS-neutralizing activity of the peptides was examined by the chromogenic *Limulus amebocyte* lysate (LAL) assay. Secondary structure of the peptides in the presence of LPS or SDS was investigated by circular dichroism (CD) spectroscopy.

Experimental Section

Materials. Fmoc (9-fluorenylmethoxycarbonyl) amino acids and Fmoc amino acid-Wang resins were purchased 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). Lipopolysaccharide (LPS, from *Escherichia coli* O111:B4), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were supplied from Sigma Chemical Co (St. Louis, MO). DMEM and fetal bovine serum (FBS) were obtained by HyClone (SeouLin, Bioscience, Korea). RAW264.7 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.

Peptide Synthesis. The peptides listed in Table 1 were prepared using the standard Fmoc-based solid-phase method. 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 TFA/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, 300 Å, 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) (Table 1).

Table 1. Amino acid sequences, calculated and observed molecular masses of LL-37 and its analogs

Peptides	Amino acid sequences	Molecular mass	
		Calculated	Observed
LL-37	LLGDFFRKSKEKIGKEFKRIVQ RIKDFLRNLVPRTES	4493.4	4493.2
a4-W1	IGKKWKRIVKRIKKFLRKL	2439.2	2438.9
a4-W2	IGKKFKRIVKRIKKWLRKL	2439.2	2438.4
a4-W1-D	IGkKWKrIVkRIkKFLrKL	2439.2	2438.4
a4-W2-D	IGkKFKrIVkRIkKWLrKL	2439.2	2438.6
a4-W1-E	igkkwkrivkrikkflrkl	2439.2	2438.8
a4-W2-E	igkkfkrivkrikkwlrkl	2439.2	2438.5

Small letters indicate D-amino acids.

Antimicrobial Activity (MIC). All bacterial strains were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The antimicrobial activity of the peptides 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.

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 1 h 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). 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:

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

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. The mean residue ellipticity, [θ], was given in deg·cm^2·dmol^{-1}: [θ] = [θ]obs (MRW/10 × l × 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: % α-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, respec-

tively.[17]

Cell Culture. RAW264.7 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, 100 μg/mL streptomycin and 25 μg/mL 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.

Cytotoxicity (MTT Proliferation Assay). Cytotoxicity of peptides against RAW264.7 cells was determined using the MTT assay.[18,19] RAW264.7 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 ºC 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 ºC. Precipitated MTT formazan was dissolved in 40 μL of 20% (w/v) SDS containing 0.01 M HCl for 2 h. Absorbance at 570 nm was measured using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA). Cell survival was expressed as a percentage of the ratio of A_{570} of cells treated with peptide to that of cells only.

Measurement of Nitric Oxide (NO) Production from LPS-stimulated RAW264.7 Cells. Nitrite accumulation in culture media was used as an indicator of nitric oxide (NO) production.[20] 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 $NaNO_2$.

Measurement of TNF-α Secretion 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 6 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).

LPS-Neutralizing Assay. The ability of the 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).[21,22] 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.

Results and Discussion

Hydrophobicity of Peptides. The retention time of peptides on a reverse-phase matrix was reported to be related to peptide hydrophobicity.[23] We compared the hydrophobicity of the peptides by measuring the retention time on the C_{18} reverse-phase HPLC column since the retention time reflected the hydrophobic interactions between the peptide and the C_{18} stationary phase (Table 2).[23]

Antimicrobial and Hemolytic Activities. We examined the antimicrobial activities of the peptides against a representative set of bacterial strains, including three gram-negative bacteria (*Escherichia coli* [KCTC 1682], *Pseudomonas aeruginosa* [KCTC 1637] and *Salmonella typhimurium* [KCTC 1926]) and three gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC 1621]). The minimal inhibitory concentration (MIC) values of designed peptides against six different bacterial strains are shown in Table 3. Both diastereomeric peptides (a4-W1-D and a4-W2-D) and enantiomeric peptides (a4-W1-E and a4-W2-E) showed almost similar antimicrobial activity compared to a4-W1 and a4-W2. These peptides (MIC: 2-4 μM) showed a 2-4-fold increased antimicrobial activity against five bacterial strains compared to parental LL-37 (MIC: 4-8 μM). The cytotoxicity of peptides toward mammalian cells 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, we introduced the minimal hemolytic concentration (MHC) defined as the peptide

Table 2. Net charge, RP-HPLC retention time and percent α-helicity of LL-37 and its analogs

Peptides	Net charge	RP-HPLC[a] retention time (min)	% α-helicity	
			0.1% LPS	30 mM SDS
LL-37	+6	32.07	91.2	67.4
a4-W1	+11	22.96	70.3	57.9
a4-W2	+11	22.54	76.1	65.6
a4-W1-D	+11	19.01	29.6	0
a4-W2-D	+11	18.31	27.9	0
a4-W1-E	+11	22.99	77.2	55.2
a4-W2-E	+11	22.47	70.4	51.9

[a]The peptides were eluted using a 60-min linear gradient of acetonitrile (0%) and water (90%) containing 0.1% trifluoroacetic acid (v/v). The data of LL-37, a4-W1 and a4-W2 were derived from Reference 13.

Table 3. Minimal inhibitory concentrations against different microbial strains of LL-37 and its analogs

Peptide	Minimal Inhibitory Concentration (MIC: μM)					
	Gram-negative bacteria			Gram-positive bacteria		
	E. coli [KCTC 1682]	*P. aeruginosa* [KCTC 1637]	*S. typhimurium* [KCTC 1926]	*B. subtilis* [KCTC 3068]	*S. epidermidis* [KCTC 1917]	*S. aureus* [KCTC 1621]
LL-37	8	8	4	8	8	4
a4-W1	4	4	2	2	4	4
a4-W2	4	4	2	2	4	2
a4-W1-D	4	4	2	2	4	4
a4-W2-D	2	2	2	2	4	4
a4-W1-E	4	4	2	2	4	4
a4-W2-E	4	4	2	2	4	4

The data of LL-37, a4-W1 and a4-W2 were derived from Reference 13.

Table 4. Prokaryotic selectivity (therapeutic index) of LL-37 and its analogs

Peptide	GM (μM)[a]	MHC (μM)[b]	Therapeutic Index (TI)[c]
LL-37	6.7	6.7	1.0
a4-W1	3.3	9.2	2.8
a4-W2	3.0	8.4	2.8
a4-W1-D	3.3	100 <	60.6
a4-W2-D	2.7	100 <	74.1
a4-W1-E	3.3	11.6	3.5
a4-W2-E	3.3	10.8	3.3

[a]The geometric mean (GM) of the MIC values against 6 bacterial strains. [b]MHC (minimal hemolytic concentration) is the peptide concentration causing 10% hemolysis. [c]The ratio of the MHC (μM) to the GM (μM). The data of LL-37, a4-W1 and a4-W2 were derived from Reference 13.

concentration that produces 10% hemolysis. Interestingly, both a4-W1-E and a4-W2-E displayed slightly lower hemolytic activity compared to a4-W1 and a4-W2 (Table 4). Both a4-W1-D and a4-W2-D did not show any hemolysis at even 100 μM, the highest concentration tested (Fig. 1 and Table 4).

Prokaryotic Selectivity (Therapeutic Index). 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.[24-26] The TI of each peptide was calculated as the ratio of the MHC (minimal hemolytic concentration) value to the GM (geometric mean of MICs against six selected microorganisms). When there was significant no hemolysis at the highest concentration tested (100 μM), 200 μM was used for the TI calculation, since the test was carried out by two-fold serial dilution. A high TI

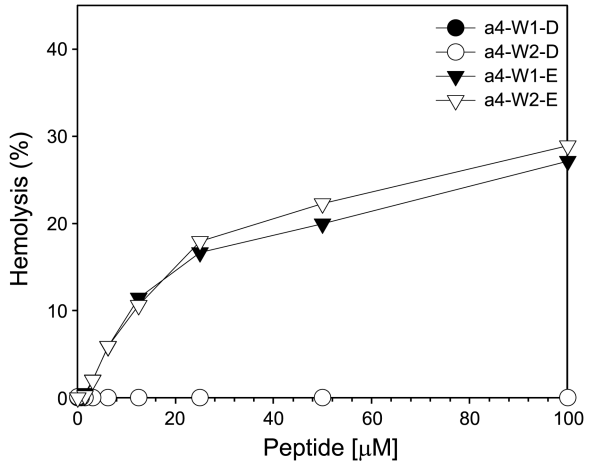

Figure 1. Hemolytic activity of the peptides against human red blood cells.

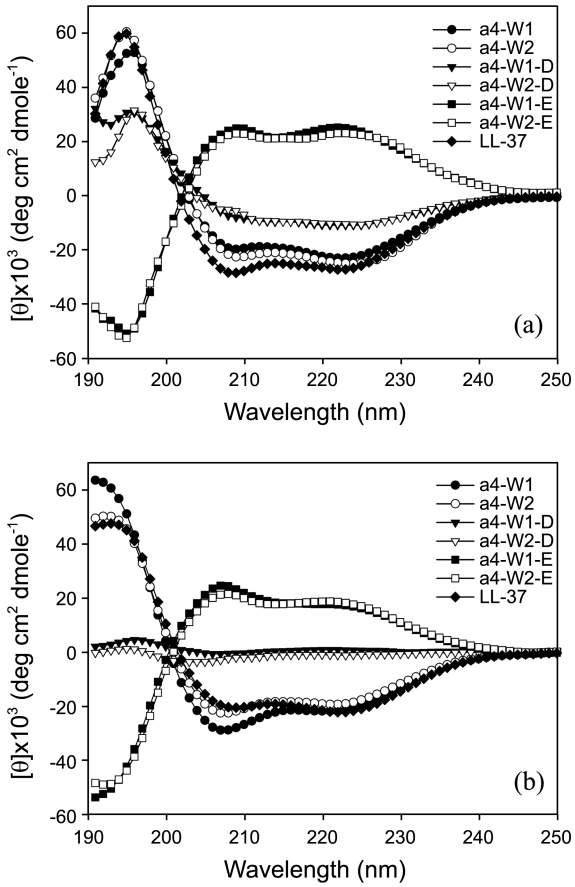

Figure 2. CD spectra of the peptides in 0.1% LPS (A) or 30 mM SDS micelles (B). CD spectra of a4-W1, a4-W2 and LL-37 in 0.1% LPS were derived from Reference 13.

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 4. Both a4-W1-D and a4-W2-D exhibited much higher prokaryotic selectivity compared to a4-W1 and a4-W2. Interestingly, both a4-W1-E and a4-W2-E had slightly higher prokaryotic selectivity compared to a4-W1 and a4-W2, since their decreased hemolytic activity.

CD Spectroscopy. Secondary structure of the peptides in 0.1% LPS or 30 mM SDS micelles was characterized by CD spectroscopy (Fig. 2). The diastereomeric peptides (a4-W1-D and a4-W2-D) exhibited no or less α-helicity in the presence of LPS or SDS compared to a4-W1 and a4-W2 due to the incorporation of the D-amino acids (Table 1). The enantiomeric peptides (a4-W1-E and a4-W2-E) had a left-handed α-helical structure, since their entire sequence is composed of only D-amino acids (Fig. 2). The α-helicity of these enantiomeric peptides was similar to that of a4-W1 and a4-W2 (Table 2).

Anti-endotoxic Activity. 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.[18,19] Both a4-W1-D and a4-W2-D were non-toxic even at 100 ìM. Similar to a4-W1 and a4-W2, both a4-W1-E and a4-W2-E were non-toxic to RAW264.7 cells until 6.25 μM, respectively, with cell viability above 90% at these concentrations (Fig. 3).[13] Therefore, all experiments using RAW264.7 macrophage cells were performed at concentrations less than 5 μM. Nitric oxide (NO) is an important inflammatory product and primarily involve in promoting inflammatory response.[27,28] TNF-α is one of the first pro-inflammatory cytokines secreted by LPS-stimulated immune cells.[9-12] To assess anti-endotoxic activity of the peptides in immune cells, we measured their ability on the inhibition of NO production and TNF-α secretion in LPS-stimulated RAW264.7 cells. As shown in Figures 4 and 5, both a4-W1-E and a4-W2-E showed potent anti-endotoxic activity comparable to that of LL-37. In contrast, both a4-W1-D and a4-W2-D displayed much less anti-endotoxic activity, since their much decreased hydrophobicity and α-helicity (Figures 4 and 5). Collectively, our results suggest that the hydrophobicity and

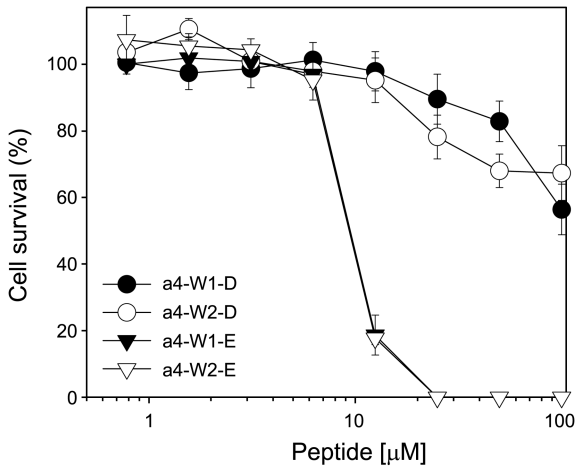

Figure 3. Cytotoxicity of the peptides against macrophage-derived RAW264.7 cells.

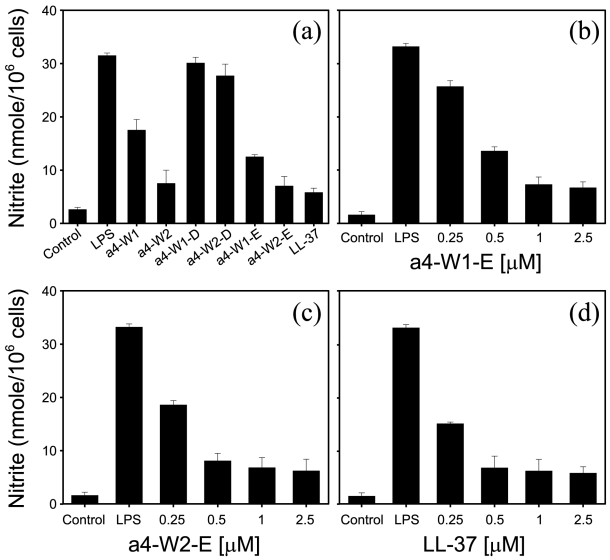

Figure 4. Effect of the peptides on NO production in LPS-stimulated RAW264.7 cells. RAW264.7 cells (5×10^5 cells/mL) were treated with 20 ng/mL LPS in the absence or presence of 0.5 μM of each peptide (a) and various concentrations (0.25 μM, 0.5 μM, 1.0 μM and 2.5 μM) of a4-W1-E (b), a4-W2-E (c) and LL-37 (d). The cell culture medium was collected, and the amount of nitrite released was measured. The error bars represent the standard deviations of the mean values determined from three independent experiments.

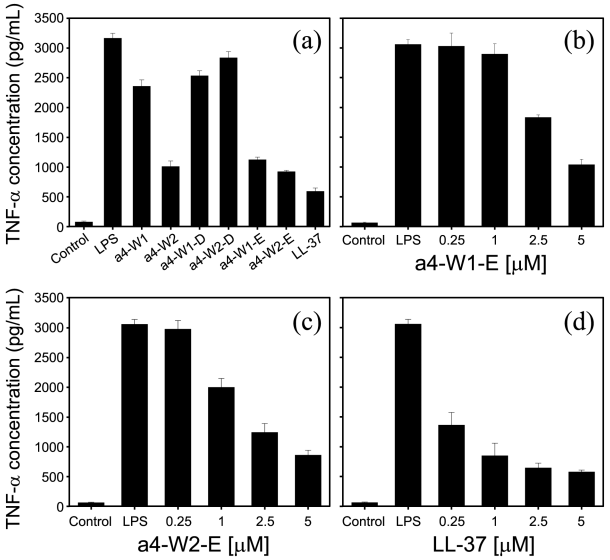

Figure 5. Effect of the peptides on TNF-α secretion in LPS-stimulated RAW264.7 cells. RAW264.7 cells (5×10^5 cells/mL) were treated with 20 ng/mL LPS in the absence or presence of 0.5 μM of each peptide (a) and various concentrations (0.25 μM, 0.5 μM, 1.0 μM and 2.5 μM) of a4-W1-E (b), a4-W2-E (c) and LL-37 (d). After incubation, the TNF-α concentration in the cell medium was evaluated using a mouse TNF-α ELISA Kit. The error bars represent the standard deviations of the mean values determined from three independent experiments.

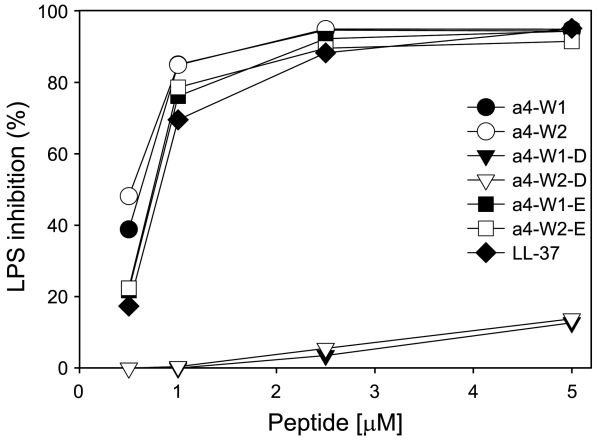

Figure 6. Dose-response curves of LPS neutralization by the peptides as determined by LAL assay. LPS (3.0 endotoxin units/mL) was incubated with four concentrations (0.5, 1.0, 2.5, and 5 μM) of each peptide for 30 min, and the amount of free LPS determined using LAL assay.

α-helicity of the peptide is important for anti-endotoxic activity.[13, 29,30]

LPS-neutralizing Activity. The limulus amoebocyte lysate (LAL) assay is an extremely sensitive indicator in the presence of free, non-neutralized LPS, allowing the detection of free LPS at the pg/mL level.[31] Limulus amoebocyte lysate (LAL) test results are generally accepted as representing the ability of a molecule to neutralize or inhibit LPS.[21,22] Thus, the ability of the peptides to neutralize LPS from *E. coli* 0111:B4 was determined by LAL assay. LAL assay was conducted at LPS concentration of 3 endotoxin units/mL, with four different concentrations of peptides, Similar to LL-37, both a4-W1-E, a4-W2-E neutralized LPS-induced activation of LAL in dose-dependent manner and neutralized almost completely endotoxin at a concentration of 2.5 μM (Fig. 6). In contrast, both a4-W1-D and a4-W2-D showed much less activity (less than 15%) at 5 μM (Fig. 6).

Protease Stability. Poor protease stability severely limits the therapeutic application of AMPs.[15,16] 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 (Fig. 7(a)) and the broth microdilution assay method (Fig. 7(b)). Trypsin treatment of a4-W1 and a4-W2 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.

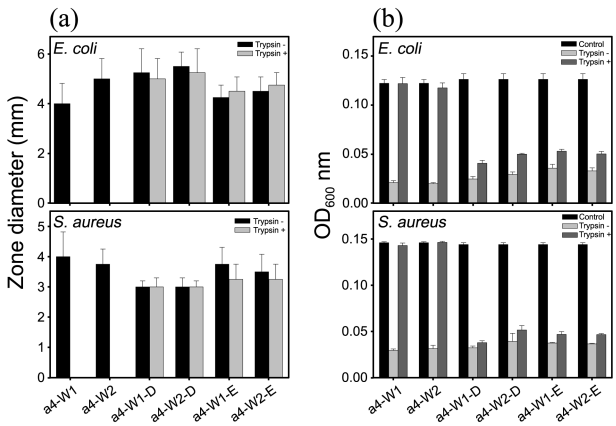

Figure 7. Inhibition of antimicrobial activity of the peptides by trypsin assessed using the radial diffusion assay (a) and broth microdilution assay (b) methods.

Conclusion

To be effective peptide antibiotic for therapeutic application, the developed AMPs must have the prokaryotic selectivity, anti-endotoxic activity and protease stability. The diastereomeric peptides (a4-W1-D and a4-W2-D) exhibited prokaryotic selectivity and protease resistance, but much less anti-endotoxic activity. In contrast, the enantiomeric peptides (a4-W1-E and a4-W2-E) had not only prokaryotic selectivity and anti-endotoxic activity but also protease stability. In particular, the enantiomeric peptides showed potent anti-endotixic and LPS-neutralizing activities comparable to that of LL-37. Taken together, both a4-W1-E and a4-W2-E holds promise as a template for the development of peptide antibiotics for the treatment of endotoxic shock and sepsis.

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