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Enantiomeric 9-mer peptide analogs of protaetiamycine with bacterial cell selectivities and anti-inflammatory activities

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Protaetiamycine is an insect defensin, derived from the larvae of the beetle *Protaetia brevitarsis*. In our previous work, we designed 9-mer peptide analogs of protaetiamycine, including 9Pbw2 (RLWLAIKRR-NH_2), 9Pbw3 (RLWLAIWRR-NH_2), and 9Pbw4 (RLWLAWKRR-NH_2). 9Pbw2 and 9Pbw4 showed high antimicrobial activity without cytotoxicity, while 9Pbw3 with higher hydrophobicity compared to 9Pbw2 and 9Pbw4 showed high cytotoxicity as well as high antimicrobial activity (Shin *et al*., *J. Pept. Sci.* 2009; 15: 559–568). In this study, we investigated the anti-inflammatory activities of 9Pbw2, 9Pbw3, and 9Pbw4 by quantitation of NO production in LPS-stimulated RAW264.7 cells. The results showed that only 9Pbw3 has strong inhibition of NO production, implying that Trp^7 as well as optimum level of hydrophobicity may play key roles in the anti-inflammatory activity of 9Pbw3. In order to design potent anti-inflammatory peptide with lower cytotoxicity as well as high stability from cleavage by protease compared to 9Pbw3, we synthesized 9Pbw3-D, the all-D-amino acid analog of 9Pbw3. 9Pbw3-D showed less cytotoxicity against RAW264.7 cells as well as considerably stronger inhibition of NO production and inflammation-induced cytokine production in LPS-stimulated RAW264.7 cells than 9Pbw3. 9Pbw3-D inhibited the gene expression of inflammatory-induced cytokine significantly more than 9Pbw3 and showed high resistance to proteolytic digestion. Binding of 9Pbw3-D with LPS caused higher enhancement of the FITC fluorescence as a result of its stronger interaction with LPS compared to that of 9Pbw3 and this result is in good agreement with their anti-inflammatory activities. 9Pbw3-D with higher anti-inflammatory activity as well as lower cytotoxicity against mammalian cell compared to 9Pbw3 can be a potent noncytotoxic antibiotic candidates. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: protaetiamycine; antimicrobial peptide; bacterial cell selectivity; anti-inflammatory activity; enantiomeric analog

Introduction

Appearance of resistant strains and cytotoxicity of antibiotic agents have necessitated the ongoing efforts to identify more potent and safer antibiotic agents. The mechanisms of actions of antimicrobial peptides are different from those of other therapeutic antibiotics and therefore antimicrobial peptides might constitute a class of attractive new drug candidates against microorganisms resistant to currently available antibiotics. Antimicrobial peptides are important components of the innate immune systems of all living organisms [1–5]. Recently, various antimicrobial peptides providing innate immunity against invading pathogens such as bacteria, fungi, and yeast have been identified from diverse species, including insect and animal species [6–8]. The innate immune systems of invertebrates, particularly of insects, have revealed the importance of antimicrobial peptides in defense. Insects are extremely resistant to bacterial infections [9]. A majority of the defensive peptides in insects are produced in either the fat bodies or hemocytes and then released into the hemolymph [10,11]. Antimicrobial peptides from insects can be divided into three categories: (i) peptides with intramolecular cysteine disulfide bonds forming hairpin-like β-sheets or α-helix/β-sheet mixed structures, (ii) peptides with amphipathic α-helical structures, and (iii) proline- or glycine-rich peptides [12,13].

In insects, the defensins constitute a large family of cationic cysteine-rich antimicrobial peptides; they are active against Gram-positive bacteria, Gram-negative bacteria and filamentous fungi [14,15]. These cysteine-rich peptides have multifunctional properties, with implications as potential therapeutic agents. We

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Abbreviations used: *CFU, colony-forming units; CH, cholesterol; EYPC, egg yolk L-α-phosphatidylcholine; EYPG, egg yolk L-α-phosphatidylglycerol; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; h-RBCs, human red blood cells; KCTC, Korean collection for type cultures; LB, Luria–Bertani; LPS, lipopolysaccharide; LUV, large unilamellar vesicle; mMIP-2, murine macrophage inflammatory protein 2; mTNF-α, murine tumor necrosis factor α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; ODS, octadecylsilica; PBS, phosphate-buffered saline; PBST, PBS in Tween 20; RPC, reversed phase chromatography; RPMI, Roswell Park Memorial Institute; RT, room temperature; RT-PCR, reverse transcription-PCR; SM, sphingomyelin; TMB, 3,3′,5,5′,-Tetramethylbenzidine.*

have previously identified a novel insect defensin, protaetiamycine from the larvae of a beetle, *Protaetia brevitarsis* [16]. Since a short antimicrobial peptide with bacterial cell selectivity would be an attractive candidate for a therapeutic agent, we designed four 9-mer analogs of protaetiamycine on the basis of its sequence from Ala^{22} to Gly [30]. Among the analogs, 9Pbw2 (RLWLAIKRR-NH_2), 9Pbw3 (RLWLAIWRR-NH_2), and 9Pbw4 (RLWLAWKRR-NH_2) show high antimicrobial activity, while only 9Pbw3 is highly cytotoxic against mammalian cell [17]. 9Pbw2 has only one Trp residues (Trp^3) and four positively charged residues. 9Pbw4 has two Trp residues (Trp^3 and Trp^6) and four positively charged residues with high bacterial cell selectivity, while 9Pbw3 with high cytotoxicity has only three positively charged residues as well as two Trp residues (Trp^3 and Trp^7), resulting in higher hydrophobicity compared to 9Pbw2 and 9Pbw4.

In this study, we measured the anti-inflammatory activities of 9Pbw2, 9Pbw3, and 9Pbw4 and found that only 9Pbw3 shows strong inhibition of NO production in LPS-stimulated RAW264.7 cells. In order to decrease the cytotoxicity of 9Pbw3 and increase the stability from cleavage by protease, we synthesized 9Pbw3-D, the all-D-amino acid analogs of 9Pbw3. Cytotoxicities and anti-inflammatory activities of 9Pbw3 and 9Pbw3-D were investigated. 9Pbw3-D showed improved anti-inflammatory activities with lower cytotoxicity compared to 9Pbw3.

Materials and Methods

Peptide Synthesis

All peptides listed in Table 1 were prepared by solid-phase synthesis using Fmoc chemistry. They were purified by RP-HPLC on a C_{18} column (20 × 250 mm; Shim-pack; Shimadzu, Kyoto, Japan), using a gradient of 20–50% acetonitrile in H_2O with 0.1% TFA for 30 min. Analytical HPLC with an ODS column (4.6 × 250 mm; Shim-pack) revealed that the purified peptides were >95% homogenous (data not shown). Furthermore, they had the correct atomic masses as determined by MALDI-TOF-MS (Table 1). The relative hydrophobicity of the peptides was determined by measuring their retention times in resource RPC (3 ml; GE Healthcare, Uppsala, Sweden), using a gradient of 0–50% acetonitrile in H_2O with 0.05% TFA for 1 h. For all experiments, the concentration of the peptides was carefully checked by UV spectrometer at 280 nm and the purity of the peptides was over 95%.

Antibacterial Activity

Escherichia coli (KCTC 1682), *Salmonella typhimurium* (KCTC 1926), *Pseudomonas aeruginosa* (KCTC 1637), *Bacillus subtilis* (KCTC 3068), *Staphylococcus epidermidis* (KCTC 1917), and *Staphylococcus aureus* (KCTC 1621) were purchased from KCTC, Taejon, Korea. MICs of peptides against the bacteria were determined by the broth microdilution method. The bacteria were grown to the mid-log phase in a medium (pH 7.0) containing 10 $g \cdot l^{-1}$ of bactotryptone, 5 $g \cdot l^{-1}$ of yeast extract, and 10 $g \cdot l^{-1}$ of NaCl. The peptides were filtered through a 0.22-μm filter and stepwise diluted in a medium of 1% bactopeptone. The test microorganism (final bacterial suspension: 2×10^6 CFU·ml^{-1}), suspended in the growth medium (100 μl), was mixed with 100 μl of twofold-diluted serial solution of each peptide in a 96-well plate with three replicates for each test sample. The plates were incubated at 37 °C for 20 h. MIC was defined as the lowest concentration of antibiotic causing complete inhibition of visible growth, compared with the growth in an antibiotic-free control well. The experiments were replicated at least three times to confirm the reproducibility of the method under the abovementioned conditions.

Hemolytic Activity

Hemolytic activities of the peptides were tested against h-RBCs. Fresh h-RBCs were washed three times with PBS (35 mM phosphate buffer containing 150 mM NaCl, pH 7.4) by centrifugation at 1000 *g* for 10 min and resuspended in PBS. 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 and the incubated samples were centrifuged at 1000 *g* for 5 min. Release of hemoglobin was monitored by measuring the absorbance of the supernatants at 405 nm. Controls for no hemolysis (blank) and 100% hemolysis consisted of h-RBCs suspended in PBS and 0.1% Triton X-100, respectively. Percentage of hemolysis was calculated on the basis of the OD of the supernatants, using the following equation:

$$\text{Hemolysis (\%)} = [(OD_{405\ nm}\ \text{sample} - OD_{405\ nm}\ \text{no-lysis control})/(OD_{405\ nm}\ \text{100\%-lysis control} - OD_{405\ nm}\ \text{no-lysis control})] \times 100.$$

Cytotoxicity Against RAW264.7 Cells

The mouse macrophage cell line RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics (100-U·ml^{-1} penicillin, 100-μg·ml^{-1} streptomycin) at 37 °C in a 5% CO_2 atmosphere. The cells were maintained in suspension or as monolayer cultures and subcultured. Percentage of growth inhibition was evaluated using an MTT assay for counting the number of viable cells. A total of 1×10^4 cells per well was seeded into a 96-well plate for 24 h, treated with various concentrations of peptides, and further incubated for 24 h at 37 °C. Subsequently, 20 μl of MTT at a concentration of 5 mg·ml^{-1} was added to each well and the cells were incubated for additional

Table 1. Amino acid sequences and properties of 9-mer peptide analogs of protaetiamycine

Peptide	Amino acid sequence	Molecular mass (Da)	Net charge	Hydrophobicity	RP-HPLC retention time (min)
9Pbw2	RLWLAIKRR-NH_2	1211.16	+5	−0.35	20.2
9Pbw3	RLWLAIWRR-NH_2	1269.19	+4	1.82	27.6
9Pbw4	RLWLAWKRR-NH_2	1283.79	+5	−0.24	21.2
9Pbw3-D	RLWLAIWRR-NH_2	1269.19	+4	1.82	27.4

Hydrophobicity is the total hydrophobicity (sum of the hydrophobicity indices of all the residues) divided by the number of residues, according to the CCS scale [18].

Journal of PeptideScience

3 h. Thereafter, absorbance was measured at a wavelength of 492 nm by using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Quantitation of NO Production in LPS-Stimulated RAW264.7 Cells

Nitrite accumulation in culture media was used as an indicator of NO production [19]. RAW264.7 cells were plated at a density of 1×10^5 cells·ml^{-1} in 96-well culture plates and stimulated with LPS (20 ng·ml^{-1}) from *E. coli* O111:B4 (Sigma, St. Louis, MO, USA) 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, 2% phosphoric acid) and incubated at RT for 5 min. NO production was estimated by measuring the absorbance at 540 nm and nitrite concentrations were determined using a standard concentration of $NaNO_2$.

Quantitation of Inflammation-Induced Cytokine (mTNF-α) Production in LPS-Stimulated RAW264.7 Cells by ELISA Assay

For the immobilization of anti-mouse mTNF-α antibody on the immunoplate, 0.2–0.8 μg·ml^{-1} solutions of the antibody in PBS were incubated overnight at RT. The plate was washed once with PBST and blocked overnight at RT with 200 μl of blocking solution (3% BSA and 0.02% NaN_3 in PBS). Subsequently, supernatants of LPS-stimulated RAW264.7 cells (20 ng·ml^{-1}) cultured in 1 and 10 μM each of 9Pbw3 and 9Pbw3-D for 18 h were added to the wells of the precoated plate and incubated for 2 h at RT. The plate was washed three times with PBST, treated with biotinylated-mTNF-α (0.4 μg·ml^{-1}) diluted in 0.1% BSA, and incubated for 2 h. The plate was rewashed three times with PBST and further incubated with streptavidin-peroxidase (0.3 μg·ml^{-1}) diluted in PBS. After washing again, SureBlue TMB peroxidase substrate (KPL Inc., Gaithersburg, MD, USA) was added. The enzyme reaction was incubated at RT for color development and terminated by the addition of 100 μl of 1 M H_2SO_4. Absorbance at 450 nm was detected using a plate reader. All values represent the mean ± standard deviation of at least three independent experiments [20].

RNA Isolation and cDNA Synthesis by RT-PCR

RAW264.7 cells were seeded in six-well plates (5×10^5 cells per well) with complete medium with 10% heat-inactivated FBS and 1% penicillin/streptomycin and cultured in CO_2 incubator for overnight. Cells were stimulated with 20 ng·ml^{-1} lipopolysaccharide in the 10-μM peptide in RPMI 1640 supplemented with 1% penicillin/streptomycin for 3 h. After the treatment, detached cells were washed once with PBS. Competitive RT-PCR was performed as previously described [21]. Total RNAs were extracted using RNeasy (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Each 5-μg RNA was used for cDNA synthesis with oligo (dT) primers and cDNAs were amplified with each following primers. mIL-1β primers were 5′-CTG TCC TGA TGA GAG CAT CC-3′ (sense), 5′-TGT CCA TTG AGG TGG AGA GC-3′ (antisense); mMIP-1 primers were 5′-ATG AAG CTC TGC GTG TCT GC-3′ (sense), 5′-TGA GGA GCA AGG ACG CTT CT-3′ (antisense); mMIP-2 primers were 5′-ACA CTT CAG CCT AGC GCC AT-3′ (sense), 5′-CAG GTC AGT TAG CCT TGC CT-3′ (antisense); mTNF-α primers were 5′-GTT CTG TCC CTT TCA CTC ACT G-3′ (sense), 5′-GGT AGA GAA TGG ATG AAC ACC-3′ (antisense); miNOS primers were 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′ (sense), 5′-GGG AGT AGC CTG TGT GCA CCT GGA A-3′ (antisense); and GAPDH primers were 5′-ACC ACA GTC CAT GCC ATC AC-3′(sense), 5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense). GAPDH was used as an internal standard. Samples were heated to 94 °C for 5 min, and cycled 25 times at 94 °C for 1 min, 59 °C for 1.5 min and 72 °C for 1 min, followed by an additional extension step of 72 °C for 5 min.

Resistance to Proteolytic Digestion

E. coli and *S. aureus* were first cultured for 18 h at 37 °C in 5 ml of LB broth and 10 μl of the cultured bacteria was then incubated in fresh LB broth for 3 h at 37 °C. The final concentration of bacterial suspension is 2×10^6 CFU·ml^{-1}. Each peptide having twofold MIC was added to trypsin in PBS and incubated at 37 °C for 6 h. The molar ratio of peptides and trypsin was 10 000 : 1. The bacterial suspension of 2×10^6 CFU·ml^{-1} in 1% bactopeptone was aliquoted at 100 μl per well, and 100 μl of the incubated peptide and trypsin solution was added to 100 μl of the bacterial suspension. Bacterial suspension without peptide and trypsin solution was examined as the control. After incubation for 18 h at 37 °C, the extent of bacterial growth was measured using the ELISA reader at 600 nm.

Calcein Leakage Assay

Calcein-entrapped LUVs composed of EYPC/SM/CH (5 : 5 : 1, w/w) and EYPC/EYPG (7 : 3, w/w) were prepared by vortexing the dried lipid in dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) [22]. The suspension was frozen-thawed in liquid nitrogen for ten cycles and extruded through polycarbonate filters (two stacked 100 nm pore size filters) by a LiposoFast extruder (Avestin, Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. Usually lipid vesicles were diluted to approximately tenfold after passing through a Sephadex G-50 column. The eluted calcein-entrapped vesicles were diluted further to the desired final lipid concentration for the experiment. The leakage of calcein from the LUVs was monitored by measuring fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm on a model RF-5301PC spectrophotometer (Shimadzu). For determination of 100% dye-release, 10% Triton-X_{100} in Tris-buffer (20 μl) was added to dissolve the vesicles. The percentage of dye-leakage caused by the peptides was calculated as follows:

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

where F is the fluorescence intensity shown by the peptides, F_0 and F_t are fluorescence intensities without the peptides and with Triton X-100, respectively.

Interaction of Papiliocin with FITC-Labeled LPS Aggregates

The interactions of peptides with FITC-conjugated LPS were studied by exciting 1.0 μM FITC-LPS at 480 nm, and monitoring the change in the emission of FITC at 516 nm in the presence of different concentrations of peptides (0.125, 0.250, 0.375, 0.500, 0.625, 1.63 and 2.63 μM). Samples were prepared in 10 mM phosphate buffer at pH 6.0.

Results and Discussion

Antimicrobial Activity

The antimicrobial activities of the peptides were examined against a representative set of bacterial strains as listed in

Table 2. Antimicrobial activity of peptides against standard bacterial strains

Bacterial strains	MIC[a] (μM)				
	9Pbw2	9Pbw3	9Pbw4	9Pbw3-D	Melittin
Escherichia coli	4.00	2.00	2.00	2.00	2.00
Pseudomonas aeruginosa	16.00	8.00	8.00	4.00	4.00
Salmonella typhimurium	4.00	8.00	2.00	2.00	4.00
Bacillus subtilis	4.00	2.00	2.00	2.00	4.00
Staphylococcus epidermidis	2.00	1.00	1.00	0.50	1.00
Staphylococcus aureus	4.00	2.00	2.00	2.00	4.00

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

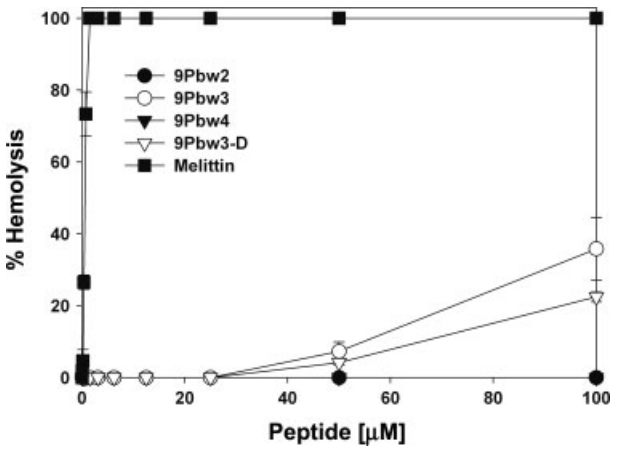

Figure 1. Dose-response curves of the hemolytic activity of peptides, 9Pbw2, 9Pbw3, 9Pbw4, and 9Pbw3-D against h-RBCs. The experiments were performed in duplicate with h-RBCs of two independent donors. The error bars represent the standard deviation values for two independent experiments.

Table 2. All the peptides demonstrated high antibacterial activity, comparable with that of melittin. 9Pbw3-D showed a little improved antibacterial activity as compared with 9Pbw3, but there were not much noticeable variations in the antibacterial activity of the D-enantiomer. Among all the peptides, 9Pbw3-D showed the highest antibacterial activity.

Hemolytic Activity and Cytotoxicity Against Mammalian Cells

We examined the hemolytic activities of the peptides two times independently. The dose-response curves for average values of the hemolytic activities of the peptides are shown in Figure 1. 9Pbw2 and 9Pbw4 with net charge of +5 did not show hemolytic activity even at 100 μM (symbols for 9Pbw2 and 9Pbw4 are overlapped). 9Pbw3 and 9Pbw3-D differ from 9Pbw2 and 9Pbw4 in hydrophobicity and retention times compare to other peptides as listed in Table 1. 9Pbw3 and 9Pbw3-D showed higher hemolytic activity than 9Pbw2 and 9Pbw4 at high concentration. 9Pbw3-D and 9Pbw3 showed hemolytic activities at 100 μM, 22.5 and 35.8%, respectively, while both peptides did not show hemolytic activities at their MIC at all.

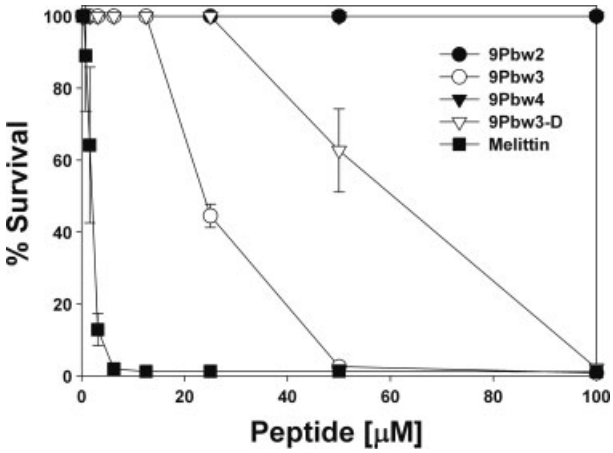

Figure 2. Dose-response curves of 9Pbw2, 9Pbw3, 9Pbw4, and 9Pbw3-D for survival of macrophage-derived RAW264.7 cells. The error bars represent the standard deviation values for two independent experiments, each performed in triplicate.

We next examined the cytotoxicities of the peptides against the mouse macrophage RAW264.7 cells two times and the data were averaged. As shown in Figure 2, 9Pbw3 showed 0% survival rate of the RAW264.7 cells at 50 μM, while 9Pbw3-D showed 60% survival rate at 50 μM. Both 9Pbw2 and 9Pbw4 showed 100% survival rate even at 100 μM (symbols for 9Pbw2 and 9Pbw4 are overlapped). These results indicate that the replacement of all the residues in the peptide 9Pbw3 with D-amino acids, yielding 9Pbw3-D, caused a decrease in cytotoxicity against RAW264.7 cells.

Inhibition of NO Production by Peptides in LPS-Stimulated RAW264.7 Cells

LPS is the major component of the outer membrane of Gram-negative bacteria. LPS, released from these bacteria during sepsis, is termed an 'endotoxin' and causes septic shock by inducing the production of high concentrations of systemic proinflammatory cytokines and NO [23]. To assess the potential anti-inflammatory activity of the peptides, we indirectly measured peptide inhibition of NO production in 20 ng·ml^{-1} of LPS-stimulated RAW264.7 macrophages by quantifying nitrite concentration. As shown in Figure 3(A), 9Pbw3 significantly inhibited NO production in the LPS-stimulated RAW264.7 macrophages at 10 μM, while 9Pbw2 and 9Pbw4 did not inhibit NO production at all. As only 9Pbw3 inhibited the NO production, we further investigated the inhibition of NO production in the LPS-stimulated RAW264.7 macrophages by 9Pbw3 and 9Pbw3-D at various concentrations, 1, 2.5, 5, 10, and 20 μM. As shown in Figure 3(B), 9Pbw3-D inhibited NO production much better than 9Pbw3 at all concentration. 9Pbw3-D inhibited NO production completely at 5 μM and 9Pbw3-D did not show any cytotoxicity against the RAW264.7 cells up to 25 μM as shown in Figure 2.

Inhibition of mTNF-α and mMIP-2 Production by Peptides in LPS-Stimulated RAW264.7 Cells

As only 9Pbw3 and 9Pbw3-D inhibited the NO production in LPS-stimulated RAW264.7 macrophages, we further investigated the inhibition of inflammation-induced cytokine production such as mTNF-α and mMIP-2 by 9Pbw3 and 9Pbw3-D in LPS-stimulated RAW264.7 cells. It was indirectly measured by quantifying mTNF-α and mMIP-2 concentrations, as shown in Figure 4(A) and (B),

Journal of PeptideScience

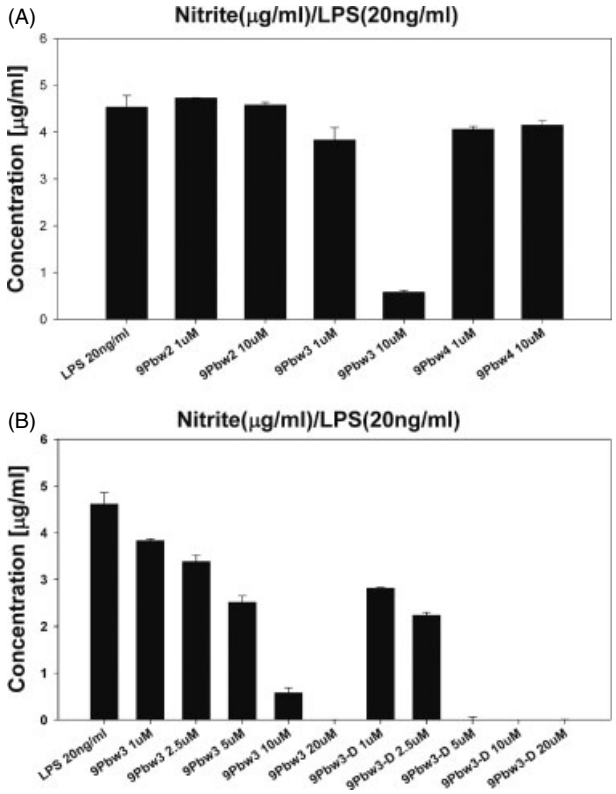

Figure 3. (A) Inhibition of NO production by 9Pbw2, 9Pbw3, and 9Pbw4 at 1 and 10 μM in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with each peptide (1 and 10 μM each) in the presence of LPS (20 ng·ml^{-1}) for 24 h. (B) Inhibition of NO production in LPS-stimulated RAW264.7 cells by 9Pbw3 and 9Pbw3-D at 1, 2.5, 5, 10, and 20 μM. RAW264.7 cells were treated with each peptide (1, 2.5, 5, 10 and 20 μM each) in the presence of LPS (20 ng·ml^{-1}) for 24 h. The error bars represent the standard deviation values for three independent experiments.

respectively. At 10 μM, 9Pbw3-D clearly inhibited both mTNF-α and mMIP-2 production in 20 ng·ml^{-1} of LPS-stimulated RAW264.7 cells and showed much higher anti-inflammatory activity than 9Pbw3.

Inhibition of Cytokine mRNA Expression by Peptides in LPS-Stimulated RAW264.7 Cells

In order to detect gene expression of inflammatory-induced cytokine, we analyzed the mRNA expression of mIL-1β, mTNF-α, miNOS, mMIP-1, and mMIP-2 by RT-PCR. The expression level of inflammatory-induced cytokine gene was decreased by 9Pbw3 and 9Pbw3-D as shown in Figure 5. 9Pbw3-D inhibited the gene expression of inflammatory-induced cytokine significantly more than 9Pbw3. These data indicate that 9Pbw3-D can be a potent anti-inflammatory agent.

Resistance to Proteolytic Digestion

Stability in serum is one of the most important requirements of a drug candidate. Antimicrobial peptides have a serious limitation for use as therapeutic agents – inactivation by protease. Trypsin is a kind of digestive protease and cleaves peptide chains at the C-terminus of the amino acid lysine or arginine. As 9Pbw3 has three arginine residues, stability against trypsin was investigated for 9Pbw3 and 9Pbw3-D. As shown in Figure 6, antimicrobial activity was observed against both *E. coli* and *S. aureus* in the bacterial suspensions not treated with trypsin, compared with the controlled bacterial suspensions. Bacterial growth was not inhibited by 9Pbw3 as compared with 9Pbw3-D. These data revealed that 9Pbw3-D was not susceptible to protease and sustained its antimicrobial activity in the presence of protease, suggesting that the all-D-amino acid peptide 9Pbw3-D can be a potential antimicrobial agent having powerful protease resistance.

Calcein Leakage Assay

To investigate the membrane-permeabilizing ability of the Pbw3 and Pbw3-D, we measured the release of the fluorescent marker calcein from liposomes of different compositions. We employed zwitterionic LUVs composed of 5 : 5 : 1 (w/w) EYPC/SM/CH and negatively charged LUVs composed of 7 : 3 (w/w) EYPC/EYPG. Figure 7 shows the dose-response relationship of the peptide-induced calcein release. 9Pbw3 and Pbw3-D permeates effectively EYPC/EYPG LUVs which mimic bacterial cell component membranes while both peptides showed lower peptide-induced calcein release

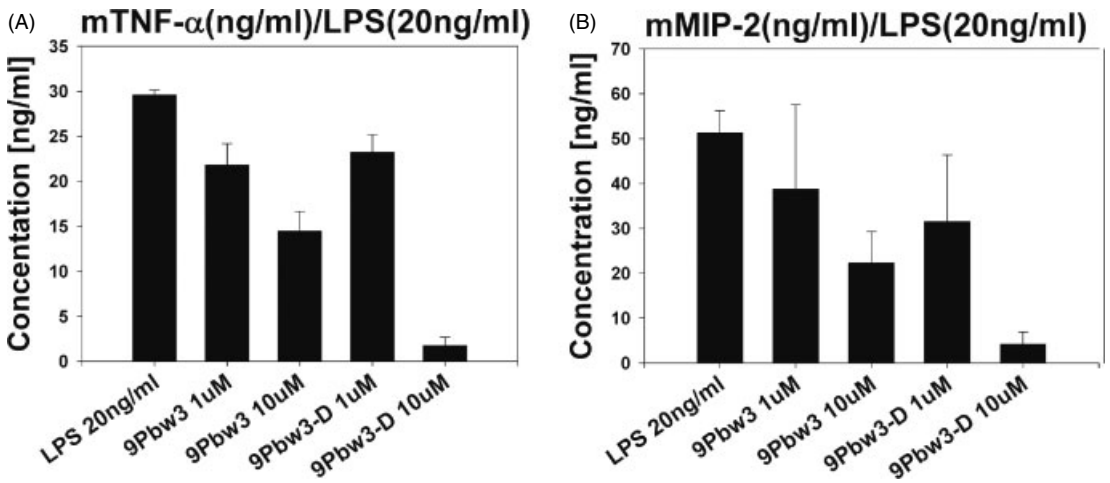

Figure 4. (A) Inhibition of mTNF-α (inflammatory cytokine) production by 9Pbw3 and 9Pbw3-D in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with each peptide (1 and 10 μM each) in the presence of LPS (20 ng·ml^{-1}) for 24 h. (B) Inhibition of mMIP-2 production by 9Pbw3 and 9Pbw3-D in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with each peptide (1 and 10 μM each) in the presence of LPS (20 ng·ml^{-1}) for 24 h. The error bars represent standard deviation values of mean determined from three independent experiments.

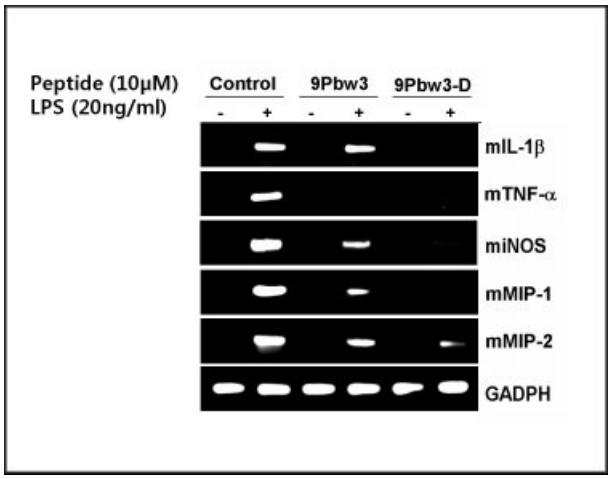

Figure 5. Effect of 9Pbw3 and 9Pbw3-D on inflammatory-induced cytokine mRNA expression by LPS-stimulated RAW264.7 cell. GAPDH was used as a control.

from EYPC/SM/CH zwitterionic vesicles which mimic components of the outer leaflet of human erythrocytes compared to negatively charged vesicles [24]. 9Pbw3-D permeated the negatively charged vesicles that mimic the bacterial cell component membranes even better at high concentration than 9Pbw3. 9Pbw3 permeated EYPC/SM/CH zwitterionic vesicles about 21% at 8 μM and 9Pbw3-D permeates about 9% at 8 μM, implying that leakage data are in good agreement with their hemolytic activities. The results suggest that the antibacterial activities of both peptides are due to permeabilization of the bacterial cell membrane and 9Pbw3-D shows higher bacterial cell selectivity compared to 9Pbw3.

FITC-Labeled LPS Aggregates

Interactions of antimicrobial peptides with LPS result in the dissociation of large LPS aggregates into smaller sizes, an effect that can be monitored as an increase in fluorescence using FITC-conjugated LPS [25,26]. Using this approach, we tested the interaction of 9Pbw3 and 9Pbw3-D with LPS. As shown in Figure 8, which represents changes in the intensity of FITC-LPS fluorescence as a function of the concentration of 9Pbw3 and 9Pbw3-D, 9Pbw3-D caused higher dose-dependent increase in FITC-LPS fluorescence than 9Pbw3, suggesting that 9Pbw3-D has stronger interaction with LPS than 9Pbw3. This data is in good agreement with higher anti-inflammatory activities of 9Pbw3-D compared to 9Pbw3.

Conclusion

In this study, we attempted to develop an antibacterial peptide with bacterial cell selectivity, anti-inflammatory properties and protease resistance by synthesizing the all-D amino acid peptide analog of 9Pbw3, a 9-mer peptide analog of protaetiamycine. In order to develop antibiotic drugs with therapeutic potential, the candidate peptide must be toxic to bacterial cells but not significantly toxic to mammalian cells. The enantiomeric analog 9Pbw3-D showed similar antibacterial activities, while 9Pbw3-D showed lower hemolytic activities as well as lower cytotoxicity against RAW264.7 cells than 9Pbw3. As listed in Table 1, 9Pbw2 has only one Trp at position 3 with a net charge of +5 and 9Pbw4 has two Trp at positions 3 and 6 with a net charge of +5, resulting in the similar hydrophobicity and retention time. 9Pbw3 has two Trp at positions 3 and 7 with a net charge of +4 resulting in higher hydrophobicity compared to 9Pbw2 and 9Pbw4. Both 9Pbw3 and 9Pbw3-D inhibited NO production significantly in LPS-stimulated RAW264.7 cells, while 9Pbw2 and 9Pbw4 did not inhibit NO production at all. These differences implied that Trp^7 in 9Pbw3 and the optimum level of hydrophobicity may be the key factors involved in the anti-inflammatory activity of 9Pbw3 and 9Pbw3-d.

Dye leakage data implied that 9Pbw3 as well as 9Pbw3-D are bacterial cell membrane targeting peptides. It has been reported that the mechanism of actions of membrane-targeting peptides include molecular interactions that are not stereospecific and thus lead to cases where enantiomeric peptides can possess equal antibacterial activity [27,28]. This suggests that peptide-mediated cell lysis involves no protein receptors on the surface of microbial membranes. However, recently, there are studies suggesting that there may be important exceptions [29]. It has been demonstrated that the lantibiotic mersacidin interacts with lipid II and interferes with transglycosylation and peptidoglycan synthesis in Gram-positive bacteria [30]. There are also studies showing different antibacterial activities for native all-L antimicrobial peptides *versus* their all-D enantiomers [31,32]. These studies suggest that receptor-type interactions may be important for some peptides on the microbial surface. Cell surface oligosaccharides with different chirality play important roles as receptor determinants in a wide

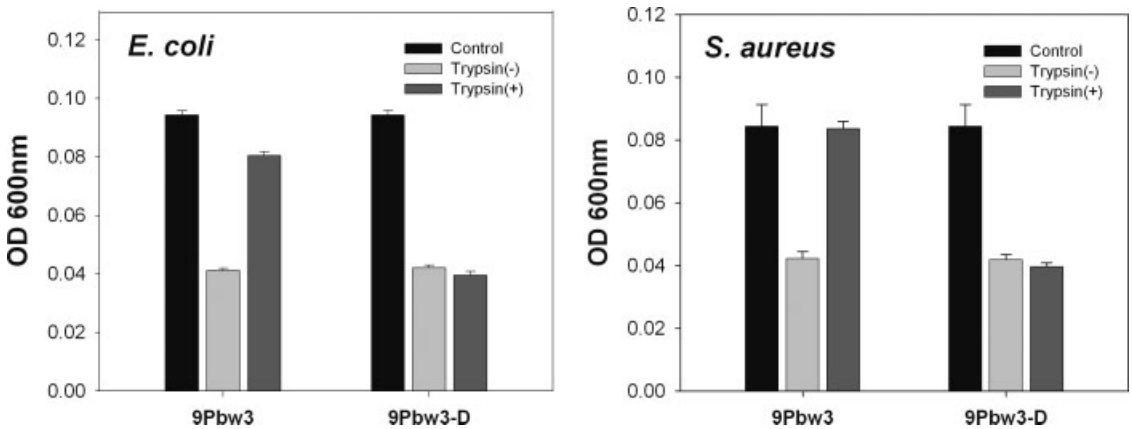

Figure 6. Inhibition of antimicrobial activity of 9Pbw3 and 9Pbw3-D by trypsin, assessed using the broth microdilution method. $OD_{600\ nm}$, optical density at 600 nm. The error bars represent standard deviation values of mean determined from three independent experiments.

Journal of PeptideScience

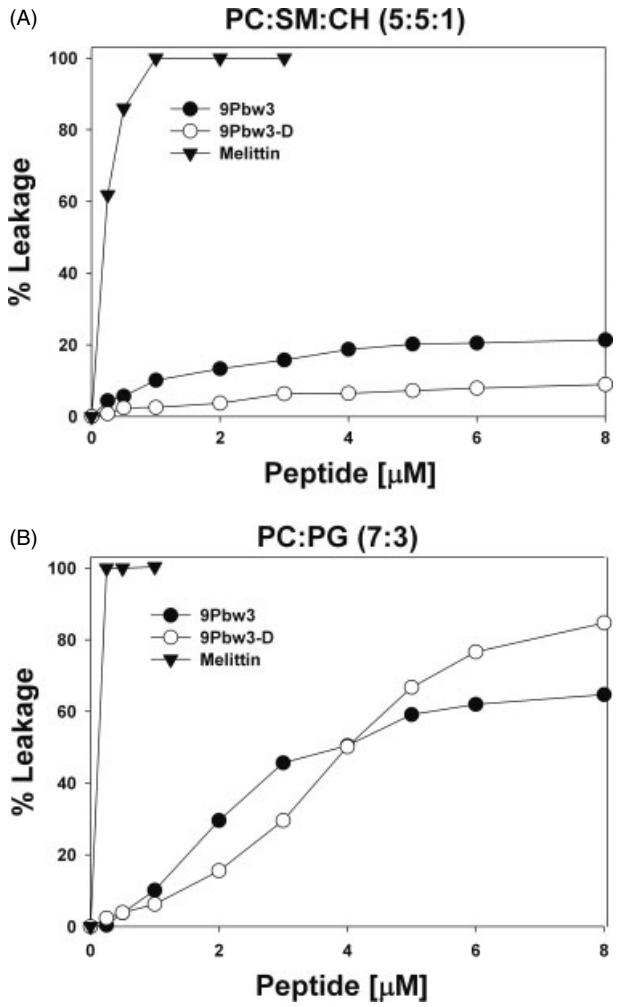

Figure 7. Dose-response curves of calcein leakage induced by the peptides from (A) EYPC/SM/CH LUVs (5 : 5 : 1, w/w) which mimic components of the outer leaflet of human erythrocytes and (B) EYPC/EYPG (7 : 3, w/w) LUVs that mimic the bacterial cell component membranes. Calcein leakage was measured 3 min after adding the peptide. The lipid concentration used in the leakage experiments was 200 μM.

range of biological recognition processes. In this study, 9Pbw3-D showed a little improved antibacterial activity as compared with 9Pbw3, but there were not much noticeable variations in the antibacterial activity of the D-enantiomer.

Compared with 9Pbw3, 9Pbw3-D inhibited very strongly NO production as well as mTNF-α and mMIP-2 production in LPS-stimulated RAW264.7 cells, which indicated that 9Pbw3-D can be a potent anti-inflammatory agent. The main component of the Gram-negative cell wall is LPS, which consists of a core region to which are attached repeating units of chiral polysaccharide moieties. LPS acts as extremely strong stimulator of macrophages, which are part of the innate immunity of diverse organisms [33–35]. In this study, changes in the intensity of FITC-LPS fluorescence as a function of the concentration of 9Pbw3 and 9Pbw3-D suggested that 9Pbw3-D has stronger interaction with LPS than 9Pbw3. As binding of peptides with LPS causes an enhancement of the FITC fluorescence as a result of the plausible dissociation of LPS aggregates, this result is in good agreement with their anti-inflammatory activities. It can be deduced that the small conformational difference in 9Pbw3 and 9Pbw3-D may

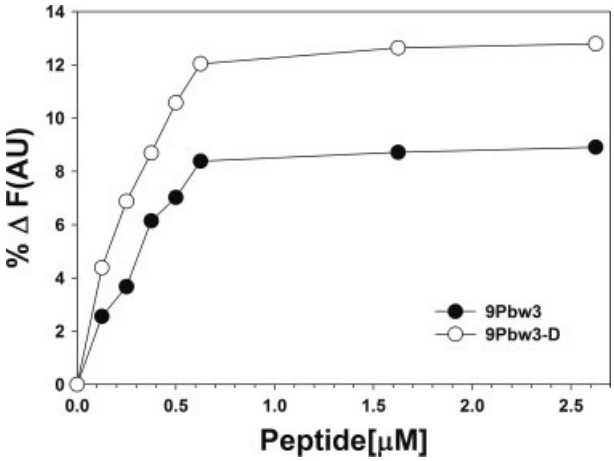

Figure 8. Enhancement of the intensity of FITC-labeled LPS as a function of 9Pbw3 and 9Pbw3-D concentration.

lead to dramatic difference in their anti-inflammatory activities and chirality-related interactions on the oligosaccharides with different chirality, resulting in improved anti-inflammatory activity compared with that of 9Pbw3. From all experimental results, it can be suggested that their chiralities are less important for the antibacterial activities, while their chiralities are more important for the anti-inflammatory activities.

Even though there were not much noticeable variations in the antibacterial activity of 9Pbw3 and 9Pbw3-D, 9Pbw3-D has lower cytotoxicity against mammalian cells and higher anti-inflammatory activity as well as its higher binding properties with LPS than 9Pbw3. Exact reason for these differences in bacterial cell selectivities and anti-inflammatory activities of 9Pbw3 and 9Pbw3-D should be investigated further. In conclusion, 9Pbw3-D showed high antibacterial and anti-inflammatory activities with bacterial cell selectivity and was resistant to proteolytic enzyme degradation, suggesting that it can indeed be a potential candidate for a therapeutic antibiotic in clinical applications.

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