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Cell Selectivity and *Anti*-inflammatory Activity of a Novel Tritrpticin Analog Containing Homo-tryptophan Peptoid Residues

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Tritrpticin (TP), a member of the cathelicidin family, is a 13-amino-acid antimicrobial peptide (AMP) with a unique amino acid sequence (VRRFPWWWPFLRR) found in porcine leukocytes.[1] TP has a high proportion of Arg (30%) and Trp (23%) residues. It forms a tritryptophan motif in the center of the peptide. Like indolicidin, it is classified into the group of Trp/Arg-rich AMPs. The solution structure of TP bound to sodium dodecyl sulfate (SDS) micelles was determined by the nuclear magnetic resonance (NMR) study.[2] TP adopts an amphipathic turn-turn structure, with the Trp residues clustered together and inserted in the hydrophobic core of the micelle.[2] TP has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as some fungi.[1] Due to its short length and broad spectrum of antimicrobial activity, TP is a promising candidate for the development of antimicrobial drugs. The primary problem associated with peptide antimicrobial drug development is its lack of cell selectivity, the ability to distinguish pathogen cell against host cell, and one solution to overcome this is *via* incorporation of peptoid residues into the peptide.[3-5]

Peptoid residues are structurally similar to amino acids, but have their side chains transferred from the α-carbon to the amide nitrogen.[6] Lacking the ability to form backbone hydrogen bonds, peptoids do not form standard peptide secondary structures. The introduction of peptoid residues in α-helical AMPs has also been reported to increase cell selectivity. In the previous study, we found that the substitution of Ala peptoid residue (*N*ala) for Leu-9 and Leu-13 of an amphipathic α-helical model peptide (KLW) (KWKK-LLKKLLKLLKKLLK-NH_2) can significantly increase the cell selectivity.[3] Similar approaches were used to enhance the cell selectivity of cathelicidin-derived Trp/Pro-rich peptides and melittin.[4,5]

In this study, to investigate the effect of peptoid residue-substitution on cell selectivity and *anti*-inflammatory activity of TP, we designed and synthesized a novel TP analog (TP-*N*htrp) in which Trp-6 and Trp-8 residues were replaced with homo- tryptophan peptoid residue (*N*htrp) (Fig. 1 and Table 1). We examined the antimicrobial activity of the peptides against a representative set of bacterial strains, including two Gram-negative bacteria (*Escherichia coli* [KCTC 1682] and *Pseudomonas aeruginosa* [KCTC 1637]) and two Gram-positive bacteria (*Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC 1621]). The MIC values of the peptides are shown in Table 2. TP and its Nhtrp-substituted analog (TP-*N*htrp) exhibited the same antimicrobial activity with MIC ranging between 4 μM and 16 μM. Next, to check the cell selectivity, we have assessed the peptide induced hemolysis in human red blood cells. Concentration-response curves for the hemolytic activity of the peptides are shown in Figure 2. A somewhat decreased hemolytic activity was observed for TP-*N*htrp, as compared with TP.

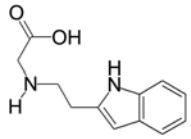

Figure 1. The chemical structure of homo-tryptophan peptoid residue (*N*htrp).

Table 1. Amino acid sequences and calculated and observed molecular masses of tritrpticin (TP) and its analog (TP-*N*htrp)

Peptides	Amino acid sequences	Molecular MS	
		Calculated	Measured[a]
TP	VRRFPWWWPFLRR	1902.3	1903.1
TP-*N*htrp	VRRFP***W***W***W***PFLRR	1930.3	1930.2

W: *N*htrp (homo-tryptophan peptoid residue). [a]Molecular masses of the peptides were determined by MALDI-TOF MS.

To determine cell selectivity of TP and TP-*N*htrp, we calculated their therapeutic index (TI), which can be a measure of the relative safety of the drug.[7] It is a measure of peptide's capability to differentiate any pathogen against host cells.[7] The TI of the peptides was calculated as the ratio of the HC_{50} (the concentration that produces 50% hemolysis against human red blood cells) to GM (geometric mean of MICs against four selected microorganisms). Larger values of TI correspond to greater cell selectivity. As shown in

Table 2. Antimicrobial activity of tritrpticin (TP) and its analog (TP-*N*htrp)

Peptide	MIC[a] [μM]				GM		
	Gram(+) bacteria		Gram(−) bacteria		Gram (+)[b]	Gram (−)[c]	Gram (+, −)[d]
	S. epidermidis	*S. aureus*	*E. coli*	*P. aeruginosa*			
TP	8	4	8	16	6	12	9
TP-*N*htrp	8	4	8	16	6	12	9

[a]MIC [μM] is defined as the lowest peptide concentration that causes 100% inhibition of microbial growth. [b]Geometric mean of MICs against two Gram-positive strains tested. [c]Geometric mean of MICs against two Gram-negative strains tested. [d]Geometric mean of MICs against all of the four strains tested.

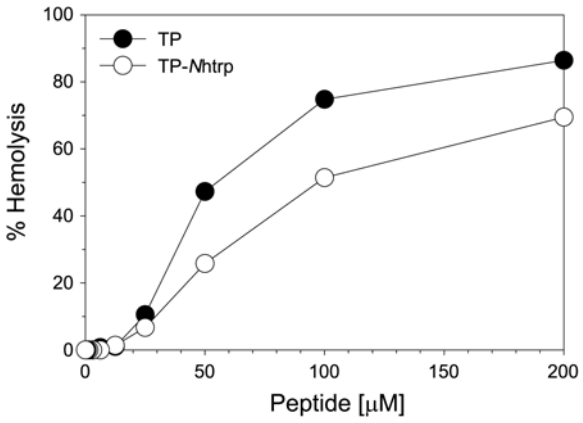

Figure 2. Concentration-response curves of the hemolytic activity of the peptides against human red blood cells.

Table 3, TP-*N*htrp showed higher cell selectivity than TP, since TP-*N*htrp caused reduced hemolysis, while retaining the antimicrobial activity.

To examine conformational differences between TP and TP-*N*htrp, the secondary structures of peptides were estimated by CD spectroscopy. In the membrane-mimicking environments such as 50% TFE (Fig. 3(a)) or 30 mM SDS micelles (Fig. 3(b)), the CD spectra of TP showed a negative peak around 225 nm, which could be caused by either the tryptophan side chains or turn structures.[8] However, the CD spectra of TP-*N*htrp displayed a very large negative peak around 190-200 nm, typical for a random coil structure.

To examine whether the cytoplasmic membrane of bacterial cells is the target of TP and TP-*N*htrp, the abilities of the peptides to depolarize the cytoplasmic membrane of Gram-positive *Staphylococcus aureus* were examined by using the membrane potential-sensitive fluorescent dye $diSC_3$-5 (Fig. 4). This dye is distributed between the cells and the medium, depending on the cytoplasmic membrane potential, and self-

Table 3. Hemolytic activity and therapeutic index of tritrpticin (TP) and its analog (TP-*N*htrp)

Peptide	HC_{50}[a] [μM]	Therapeutic index (TI)[b]		
		Gram (+)	Gram (−)	Gram (+, −)
TP	55	9.2	4.6	6.1
TP-*N*htrp	96	16	8	10.7

[a]HC_{50} displays the peptide concentration that causes 50% hemolysis. [b]The therapeutic index (TI) is defined as the ratio HC_{50}/GM.

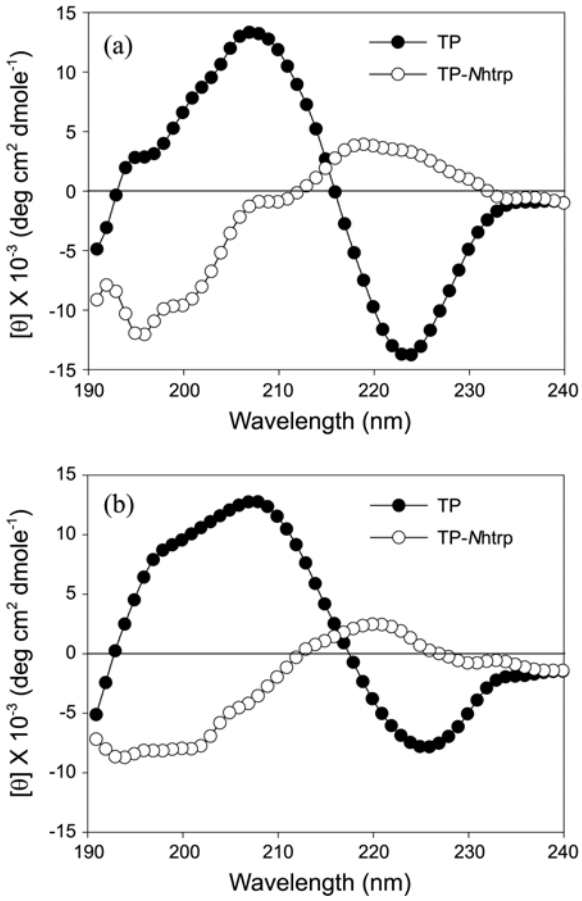

Figure 3. CD spectra of TP and TP-*N*htrp in 50% TFE (a) or 30 mM SDS micelles (b).

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. Both TP and TP-*N*htrp at their MIC (4 μM) caused a collapse in membrane potential within 1 min as significant as 100% of the maximum fluorescence recovered by the addition of gramicidin D (Fig. 4). This result suggested that the major target of TP and TP-*N*htrp is the cytoplasmic membrane of bacterial cells.

Lipopolysaccharide (LPS) is the major molecular component of the outer membrane of Gram-negative bacteria.[9] As a potent inducer of the innate immune system, LPS activates monocytes and macrophages to secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), inter-

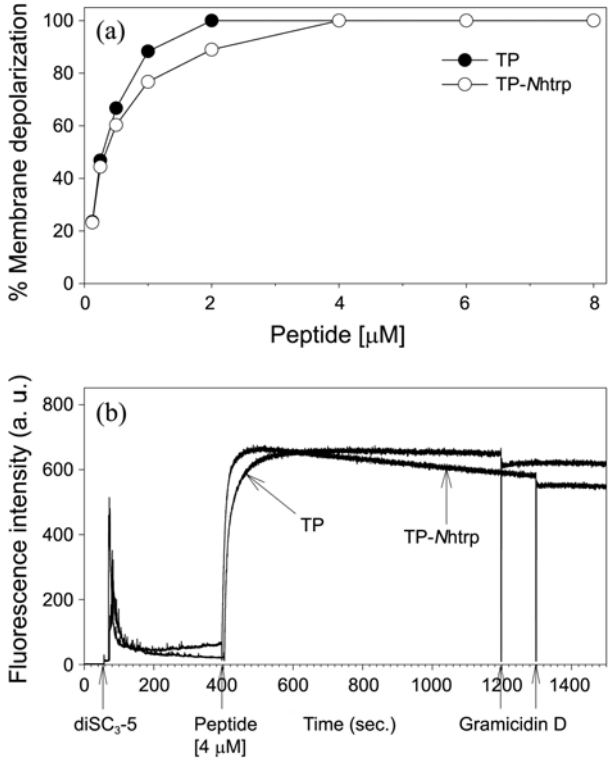

Figure 4. Concentration-dependent membrane depolarization of *Staphylococcus aureus* (OD_{600} = 0.05) by the peptides using the membrane potential-sensitive dye, $DiSC_3$-5. (b) Time-dependent membrane depolarization of *Staphylococcus aureus* by the peptides (peptide concentration: 4 μM).

leukin-6 (IL-6), and interleukin-1β (IL-1β), and induce other inflammatory mediators, such as nitric oxide (NO).[10,11] Many clinically used antibiotics cause the release of LPS from the microbial cell wall and give rise to a prolonged activation of the immune cells, which results in an unbalanced and over-production of cytokines.[12] This can rapidly lead to constellation of symptoms termed endotoxin shock or septic shock, which, in extreme cases, may lead to death.[11] Therefore, an effective antimicrobial agent should not only exert antimicrobial activity but also have the ability to neutralize LPS. Recent studies have shown that in addition to antimicrobial action, a few cathelicidin family antimicrobial peptides such as LL-37, indolicidin, bactenecin, and β-defensin also have the potential to neutralize LPS-induced cellular cytokine and/or NO release.[13,14]

Therefore, to investigate the effect of homo-tryptophan peptoid residue (*N*htrp)-substitution on *anti*-inflammatory activity of TP, we assessed the ability of the peptides to inhibit nitric oxide (NO) release in LPS-stimulated mouse macrophage RAW264.7. As shown in Figure 5, both TP and TP-*N*htrp significantly inhibited NO production in LPS-simulated RAW264.7 macrophage cells at the concentration of 10 μM, compared with LPS-stimulated cells alone. TP-*N*htrp had higher potency to inhibit LPS-induced NO production from macrophages when compared to TP.

To examine whether the improved *anti*-inflammatory activity of TP-*N*htrp is correlated to its increase in LPS-neutralizing

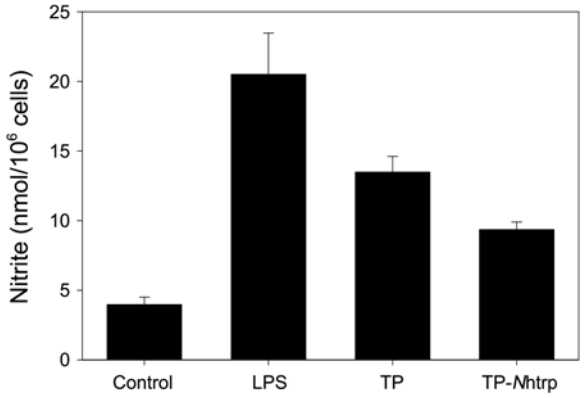

Figure 5. Inhibition of nitric oxide (NO) production by the peptides in LPS-stimulated mouse macrophage RAW264.7 cells. RAW264.7 cells (5×10^5 cells/mL) were treated with 20 ng/mL LPS in the absence or the presence of the peptides (10 μM) for 24 h. The cell culture media were then collected, and the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

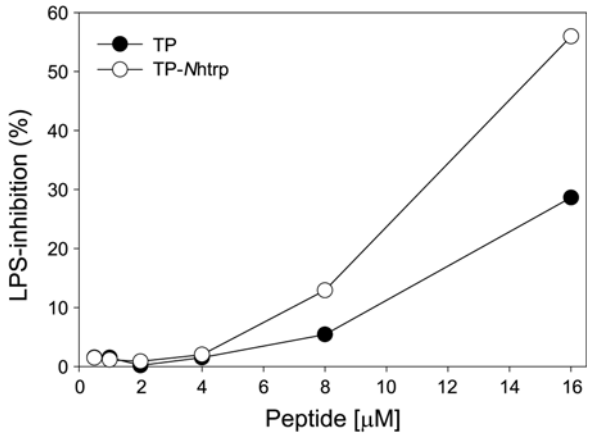

Figure 6. Concentration-response curves of LPS-neutralization by the peptides as determined by LAL assay. LPS (3.0 endotoxin units/mL) was incubated with 6 concentrations (0.5, 1, 2, 4, 8 and 16 μM) of each peptide for 30 min, and the amount of free LPS determined using LAL assay.

activity, we investigated the ability of the peptides to neutralize LPS from *E. coli* 0111:B4 by limulus amoebocyte lysate (LAL) assay. LAL assay is generally accepted as a method that can reliably measure the ability of a molecule to neutralize LPS.[15] TP-*N*htrp showed higher LPS-neutralizing activity as compared to TP (Fig. 6). This result suggested that improved *anti*-inflammatory activity of TP-*N*htrp may be due to its increase in LPS-neutralizing activity.

In conclusion, we successfully designed a novel *N*htrp-substituted TP analog (TP-*N*htrp) with improved cell selectivity and *anti*-inflammatory activity as compared to TP. Membrane depolarization suggested that the major target of TP and TP-*N*htrp is the bacterial plasma membrane. Taken together, a novel *N*htrp-substituted TP analog, TP-*N*htrp, have the potential for future development as novel antimicrobial and *anti*-inflammatory peptides.

Experimental Section

Peptide Synthesis. TP and TP-*N*htrp were synthesized by solid phase method using Fmoc-amino acids. Fmoc-*N*htrp-OH for the synthesis of TP-*N*htrp was prepared according to the method described in our previous study.[16] Purication by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) gave nal products deemed > 95% pure by analytical RP-HPLC. The success of the synthesis of the peptide was conrmed by analysis using a matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrometry (MS) (Table 1).

Antimicrobial Activity. The antimicrobial activity of the peptides was examined by the broth microdilution method described in our previous study.[17] Aliquots (100 μL) of a bacterial suspension at 2×10^6 colony-forming units (CFU)/μL 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 ºC, 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. All bacterial strains were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Hemolytic Activity. Fresh human red blood cells (hRBCs) were centrifuged, washed three times with PBS, dispensed into 96-well plates as100 μL of 4% (v/v) hRBC in PBS, and 100 μL of peptide solution was added to each well. Plates were incubated for 1 h at 37 ºC, then centrifuged at 1000 × g for 5 min. Samples (100 μL) of supernatant were transferred to 96-well plates and hemoglobin release was monitored by measuring absorbance at 414 nm. Zero hemolysis was determined in PBS (A_{PBS}) and 100% hemolysis was determined in 0.1% (v/v) Triton X-100 (A_{triton}). The hemolysis percentage hemolysis was calculated as: % hemolysis = $100 \times [(A_{sample} - A_{PBS}) / (A_{Triton} - A_{PBS})]$

Circular Dichroism (CD) Spectroscopy. The CD spectrum of the peptide was obtained with a Jasco J-715 CD spectrophotometer (Tokyo, Japan) at 25 ºC using a fused quartz cell with a 1-mm path length over a wavelength range of 190-250 nm at 0.1 nm intervals (speed, 50 nm/min; response time, 0.5 s; bandwidth, 1 nm). CD spectra were collected and averaged over three scans. Samples were prepared by dissolving the peptide to a nal concentration of 100 μg/mL in 50% (v/v) trifluoroethanol (TFE) or 30 mM sodium dodecyl sulfate (SDS).

Membrane Depolarization. *Staphylococcus aureus* [KCTC 1621] grown at 37 ºC 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. Membrane depolarization was monitored by recording changes in the intensity of fluorescence emission of the membrane potential-sensitive dye, $diSC_3$-5 ($\lambda_{ex.}$ = 622 nm, $\lambda_{em.}$ = 670 nm) after peptide addition. The membrane potential was fully dissipated by adding gramicidin D (final concentration of 0.2 nM). The membrane potential dissipating activity of the peptides is calculated as follows:

$$\% \text{ Membrane depolarization} = 100 \times [(F_p - F_0) / (F_g - F_0)]$$

where F_0 denotes the stable fluorescence value after the addition of the $diSC_3$-5 dye, F_p denotes the fluorescence value 5 min after peptide addition, and F_g denotes the fluorescence signal after gramicidin D addition.

Nitric Oxide (NO) Production from LPS-Stimulated RAW 264.7 Cells. Measurement of nitrite accumulation into the culture medium was used to determine NO production. At indicated time points, the culture medium was collected and nitrite was measured by Griess reaction.[18]

LPS-Neutralizing Assay. The ability of the peptides to neutralize LPS was measured using the commercially available Limulus amebocyte lysate (LAL) assay kit (Kinetic-QCL 1000 kit; BioWhittaker, Walkersville, MD, USA) according to the manufacturer's instruction.[15]

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