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Prokaryotic Selectivity, Bactericidal Mechanism and LPS-neutralizing Activity of Lys-linked Dimeric Peptide of Indolicidin C-terminal Hexapeptide

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Indolicidin (Ind) is a cationic antimicrobial peptide (AMP) derived from the granules of bovine neutrophils.[1] It is a short, 13 amino acid peptide (ILPWKWPWWPWRR-NH_2) that is amidated at the C-terminus and contains the highest proportion of Trp and Pro residues.[1] Like many other AMPs, it exhibits a broad spectrum of antimicrobial activity against bacteria, fungi and protozoa.[2] It has been shown that Ind adopts a disordered conformation in solution and a membrane-interacting structure containing coils and turns.[3] However, Ind exhibits some toxicity to human erythrocytes and mouse T lymphocytes.

In the present study, to investigate the effect of Lys-linked dimerization of Ind C-terminal hexapeptide (WWPWRR-NH_2) on prokaryotic selectivity, bactericidal mechanism and LPS-neutralizing activity and to develop a novel short AMP having prokaryotic selectivity and LPS-neutralizing activity, we synthesized a Lys-linked dimeric peptide (di-Ind-6) of Ind C-terminal hexapeptide (Ind-6) (Table 1). We examined the antimicrobial activity 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 MIC values of the peptides are shown in Table 2. A Lys-linked dimeric peptide (di-Ind-6) displayed 2- to 4-fold increased antimicrobial activity compared to monomeric peptide (Ind-6). Furthermore, Di-Ind-6 displayed a 2-fold enhanced antimicrobial activity against Gram-negative bacteria as compare to Ind. Next, the cytotoxicity of the peptides to mammalian cells was determined by measuring their hemolytic activity toward human red blood cells (h-RBCs) Concentration-response curves for the hemolytic activity of the peptides are shown in Figure 1. For a quantitative measure of the hemolytic activity of the peptides, we introduced the hemolytic concentration (HC_{50}) defined as the lowest peptide concentration that produces 50% hemolysis (Table 3). The HC_{50} value for Ind and di-Ind-6 was 160 μg/mL and 192 μg/mL, respectively. Di-Ind-6 was somewhat less hemolytic than Ind. In contrast, Ind-6 did not induce hemolysis even at the highest peptide concentration tested (200 μg/mL).

Table 1. Amino acid sequences and calculated and observed molecular masses of the peptides

Peptides	Amino acid sequences	Molecular MS	
		Calculated	Measured[a]
Indolicidin (Ind)	ILPWKWPWWPWRR-NH_2	1906.3	1906.0
Ind-6	WWPWRR-NH_2	985.2	985.8
di-Ind-6	$(WWPWRR)_2K$-NH_2	2081.5	2082.2

[a]Molecular masses were determined by MALDI-TOF MS.

The therapeutic potential of peptide antimicrobial drugs lies in their prokaryotic selectivity to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells such as human red blood cells. To determine prokaryotic selectivity of the peptides, we calculated their therapeutic index (TI) (Table 3). The TI is a widely employed parameter to represent the specificity of antimicrobial agents. The TI of the peptides is calculated as the ratio of the HC_{50} to GM (geometric mean of MICs against six selected

Table 2. Antimicrobial activity against bacterial strains of the peptides

Peptide	MIC[a] (μg/mL)					
	Gram-negative bacteria			Gram-positive bacteria		
	E. coli	*P. aeruginosa*	*S. typhimurium*	*B. subtilis*	*S. epidermidis*	*S. aureus*
Ind	32	32	16	16	16	8
Ind-6	64	32	32	32	32	16
di-Ind-6	16	16	8	16	16	8

[a]MIC (μg/mL) is defined as the lowest peptide concentration that causes 100% inhibition of microbial growth.

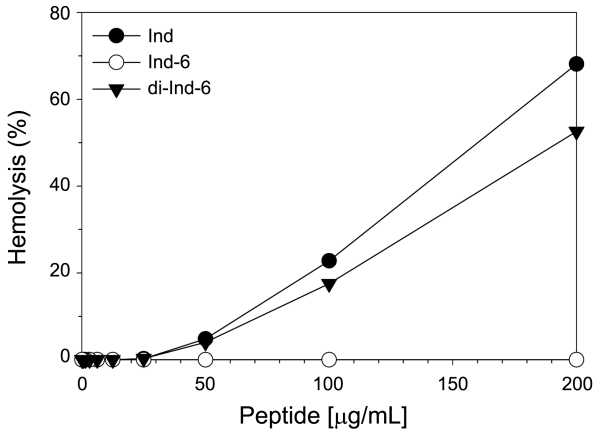

Figure 1. Concentration-response curves of the hemolytic activity of the peptides against human red blood cells.

Table 3. Therapeutic index (prokaryotic selectivity) of the peptides

Peptide	GM[a] (μg/mL)	HC_{50}[b] (μg/mL)	(TI)[c] (HC_{50}/GM)
Ind	20	160	8
Ind-6	34.7	200 <	11.5
di-Ind-6	13.3	192	14.4

[a]Geometric mean (GM) of MICs against six bacterial strains tested. [b]HC_{50} displays the peptide concentration that causes 50% hemolysis. [c]The therapeutic index (TI) is defined as the ratio HC_{50}/GM.

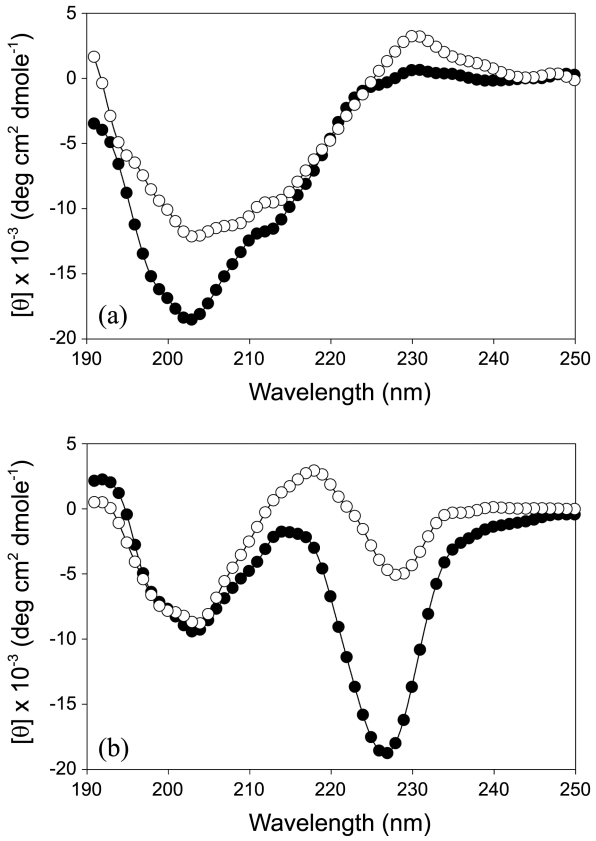

Figure 2. CD spectra of Ind and di-Ind-6 in 10 mM sodium phosphate buffer (pH 7.4) (a) or 30 mM SDS micelles (b). Symbols: Ind (●) and di-Ind-6 (○).

microorganisms); thus, larger values of TI indicate greater prokaryotic selectivity. When there was significant no hemolysis at the highest concentration tested (200 μg/mL), 400 μg/mL was used for the TI calculation, since the test was carried out by two-fold serial dilution. As shown in Table 3, Di-Ind-6 showed somewhat higher prokaryotic selectivity compared to native Ind.

To examine conformational differences between Ind and di-Ind-6, the secondary structures of peptides were estimated by CD spectroscopy (Fig. 2). The CD spectra of Ind and di-Ind-6 displayed a very large negative peak around 200 nm, typical for a random coil structure in aqueous condition (10 mM sodium phosphate buffer, pH 7.4) (Fig. 2(a)). In 30 mM SDS (sodium dodecyl sulfate) micelles (membrane-mimicking environment), the CD spectra of Ind and di-Ind-6 exhibit a large negative band centered at 227 nm (Fig. 2(b)). Such a band around 230 nm was shown to arise due to the interaction of the aromatic rings of Trp with the peptide backbone and stacking of the Trp rings.[4] CD spectra showing the large negative band at 227 nm have been suggested to represent mainly turn structure in Trp-rich peptides.[4]

Bacterial killing effect of the majority of AMPs is considered to be due to their action on the lipid matrix of bacterial cell membranes, either by forming pores, thinning the membrane or destabilizing the bilayer (*i.e.*, the membrane-targeting AMPs).[5] These cause the lysis of bacterial cells as a result of increased permeability. In contrast, a few peptides such as buforin-II were known to penetrate microbial cell membranes without inducing membrane permeabilization and cause bacterial cell death by inhibiting protein, DNA or RNA synthesis (*i.e.*, the intracellular-targeting AMPs).[6] Mechanisms of antimicrobial action of Ind remain poorly understood. Some investigations have pointed to cellular membrane permeabilization by Ind as a predominant mode of its membrane activity.[7]

To investigate the bactericidal mechanism of di-Ind-6 in more detail, we examined its ability to depolarize the cytoplasmic membrane of intact *S. aureus* (Fig. 3) and to cause the leakage of a fluorescent dye entrapped within negatively charged phosphatidylethanolamine (PE)/phosphatidylglycerol (PG) (7:3, w/w) large unilamellar vesicles (LUVs), which mimics bacterial outer membranes (Fig. 4). In membrane depolarization assay, we investigated the ability of di-Ind-6 to depolarize the cytoplasmic membrane of *S. aureus* was examined by using the membrane potential-sensitive fluorescent dye $diSC_3$-5. 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. Both Ind and di-Ind-6 caused a significant membrane depolarization in a concentration-dependent manner (Fig. 3(a)). These peptides at their MIC (8 μg/mL) 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. 3(b)). In the dye leakage assay,

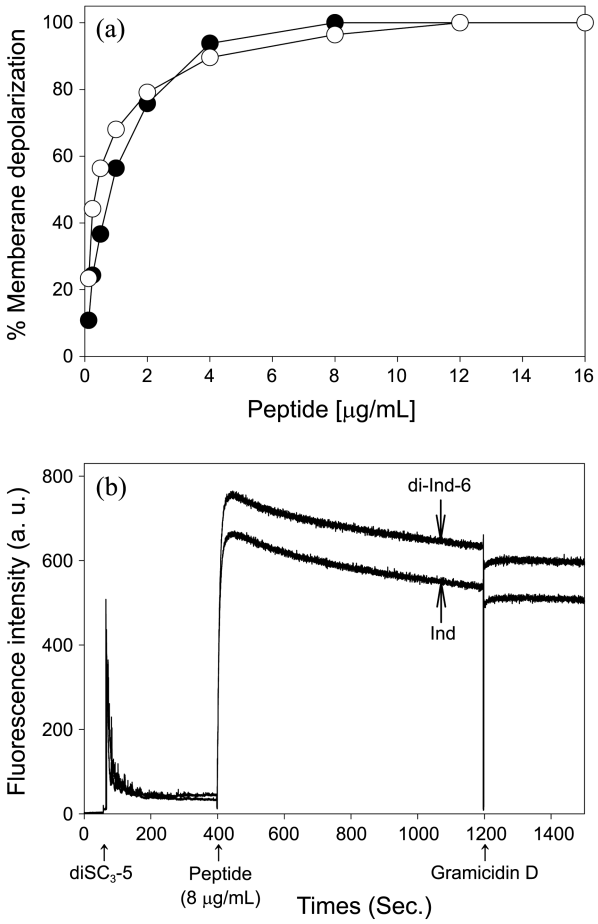

Figure 3. (a) Concentration-dependent membrane depolarization of *Staphylococcus aureus* (KCTC 1621) (OD_{600} = 0.05) by Ind and di-Ind-6. Symbols: Ind (●) and di-Ind-6 (○). (b) Time-dependent membrane depolarization by Ind and di-Ind-6 at 8 μg/mL.

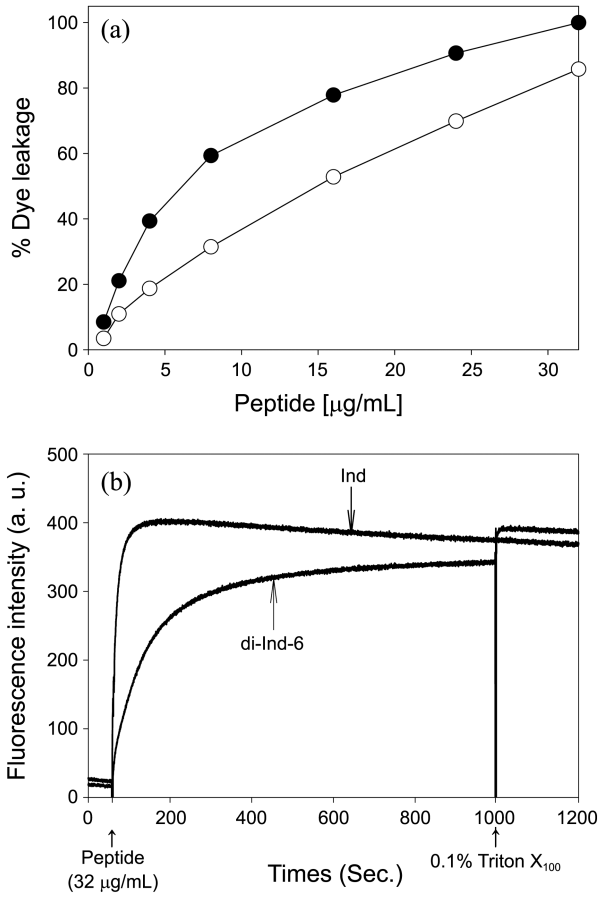

Figure 4. (a) Concentration-dependent peptide-induced dye leakage from calcein-entrapped negatively charged PE/PG (7:3, w/w) LUVs. Symbols: Ind (●) and di-Ind-6 (○). (b) Time-dependent peptide-induced dye leakage from calcein-entrapped negatively charged PE/PG (7:3, w/w) LUVs at 32 μg/mL.

both Ind and di-Ind-6 induced a significant dye leakage from PE/PG (7:3, v/v) LUVs in a concentration-dependent manner (Fig. 4(a)). These peptides led to near-complete dye (above 85%) leakage at 32 μg/mL (Fig. 4(b)). These results suggest that both Ind and di-Ind-6 kill microorganisms *via* the formation of pore/ion channels on bacterial cell membranes or the membrane disruption/perturbation.

Lipolysaccharide (LPS) 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. Recent studies have demonstrated that in addition to their antimicrobial activities, some AMPs including LL-37, Ind and linear bactenecin analog have the potential to inhibit LPS-induced cellular cytokine and/or nitric oxide (NO) release by binding directly to LPS or by blocking the binding of LPS to LPS-binding protein (LBP).[8] Therefore, an effective antimicrobial agent should not only exert prokaryotic selectivity, but must also have the ability to sequester LPS and ameliorate its toxicity. To investigate whether di-Ind-6 may have LPS-neutralizing activity, as well as potent antimicrobial activity, we assessed its ability to inhibit nitric oxide (NO) release in LPS-stimulated mouse macrophage RAW264.7 cells. Like Ind, di-Ind-6 significantly inhibited

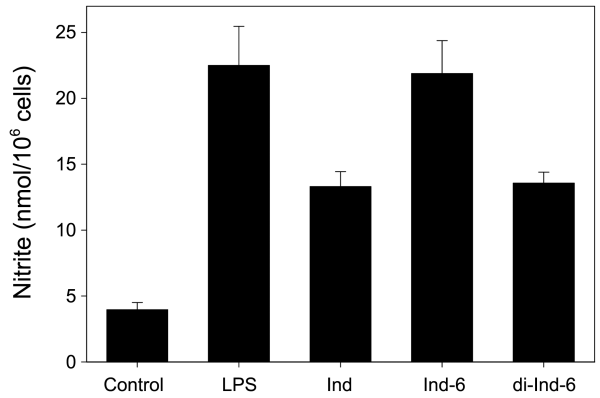

Figure 5. Inhibition of nitric oxide (NO) production by the peptides in LPS-stimulated mouse macrophage RAW264.7 cells. RAW264.7 cells (5×10^5 cells/mL) were treated with 20 ng/mL LPS in the absence or the presence of the peptides (10 μg/mL) for 24 h.

NO production in LPS-simulated RAW264.7 macrophage cells at the concentration of 10 μg/mL (Fig. 5). Di-Ind-6 showed potent LPS-neutralizing activity comparable to that of native Ind (Fig. 5).

Collectively, our results suggest that di-Ind-6 with pro-

karyotic selectivity and LPS-neutralizing activity can serve as a promising template for developing therapeutic agents for endotoxic shock and bacterial infections.

Experimental Section

Peptide Synthesis. The peptides were synthesized by solid phase peptide synthesis. Di-Ind-6 [$(WWPWRR)_2K\text{-}NH_2$] was prepared by the coupling of Fmoc-Lys(Fmoc)-OH to Rink amide MBHA resin and synthesizing simultaneously from both the α- and ε-amino groups to give two same sequence. The success of the synthesis of the peptide was confirmed by analysis using a MALDI-TOF MS (Table 1).

Antimicrobial Activity (MIC). The minimal inhibitory concentration (MIC) of the peptides against six bacterial strains was examined by the broth microdilution method as reported earlier.[9] 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 as 100 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 °C, then centrifuged at 1000 × g for 5 min. Samples (100 μL) of supernatant were transferred to 96-well plates and hemoglobin release was monitored by measuring absorbance at 414 nm. Zero hemolysis was determined in PBS (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 °C using a fused quartz cell with a 1-mm path length over a wavelength range of 190-250 nm at 0.1 nm intervals. CD spectra were collected and averaged over three scans. Samples were prepared by dissolving the peptide to a final concentration of 100 μg/mL in 10 mM sodium phosphate buffer (pH 7.4) or 30 mM SDS.

Membrane Depolarization. *S. aureus* [KCTC 1621] 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. 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 (0.2 nM).

Dye Leakage. Calcein-entrapped LUVs composed of PE/PG (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). The suspension was subjected to ten 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., Ottawa, Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. 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.

Measurement of Nitric Oxide (NO) Release from LPS-induced RAW264.7 Cells. RAW 264.7 cells were plated at 5×10^5 cells/well in 96-well plates and then incubated with LPS (20 ng/mL) in the presence of peptides. The cells with and without LPS addition were taken for maximum and basal level of nitric oxide production, respectively. The nitric oxide production was measured by using Griess reagent by reading absorbance at 540 nm as reported earlier.[10]

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