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Effects of C-Terminal Residues of 12-Mer Peptides on Antibacterial Efficacy and Mechanism

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The development of new antimicrobial agents is essential for the effective treatment of diseases such as sepsis. We previously developed a new short peptide, Pap12-6, using the 12 N-terminal residues of papiliocin, which showed potent and effective antimicrobial activity against multidrug-resistant Gram-negative bacteria. Here, we investigated the antimicrobial mechanism of Pap12-6 and a newly designed peptide, Pap12-7, in which the 12th Trp residue of Pap12-6 was replaced with Val to develop a potent peptide with high bacterial selectivity and a different antibacterial mechanism. Both peptides showed high antimicrobial activity against Gram-negative bacteria, including multidrug-resistant Gram-negative bacteria. In addition, the two peptides showed similar anti-inflammatory activity against lipopolysaccharide-stimulated RAW 264.7 cells, but Pap12-7 showed very low toxicities against sheep red blood cells and mammalian cells compared to that showed by Pap12-6. A calcein dye leakage assay, membrane depolarization, and confocal microscopy observations revealed that the two peptides with one single amino acid change have different mechanisms of antibacterial action: Pap12-6 directly targets the bacterial cell membrane, whereas Pap12-7 appears to penetrate the bacterial cell membrane and exert its activities in the cell. The therapeutic efficacy of Pap12-7 was further examined in a mouse model of sepsis, which increased the survival rate of septic mice. For the first time, we showed that both peptides showed anti-septic activity by reducing the infiltration of neutrophils and the production of inflammatory factors. Overall, these results indicate Pap12-7 as a novel non-toxic peptide with potent antibacterial and anti-septic activities via penetrating the cell membrane.

Keywords: Antimicrobial peptide, sepsis, bacterial cell selectivity, anti-inflammation, antibacterial mechanism

Introduction

In the face of continuously emerging pathogenic bacteria that are resistant to antibiotics, the development of new antibiotics and antibiotic alternatives is an inevitable and urgent task [1–4]. In particular, infectious diseases caused by multidrug-resistant bacteria, especially sepsis, are a serious threat requiring an urgent solution [4–6]. Antimicrobial peptides (AMPs), which have excellent antimicrobial activity [7–9], have been recognized as a potential solution to this challenge.

AMPs exhibit their activity through interaction with the cell membrane of pathogens [10] as a natural antimicrobial defense mechanism; thus, these peptides can also be exploited to effectively deliver a therapeutic compound in a targeted manner [10–12]. Accordingly, comprehensive evaluation of the differences in the interaction of various peptides with membranes [10] can promote their appropriate application in the treatment of diseases.

AMPs are diverse with various mechanisms of action, including decomposing or breaking membranes via a barrel-stave model [13], toroidal pore model [14], and carpet model [14, 15], or by passing through membranes to exert antibacterial activity inside the cell [16]. For example, cecropin peptides show amphipathic activity, mainly acting directly on the bacteria membrane to exert their

antibacterial effect [17, 18]. Representative peptides of the barrel-stave pore model are alamethicin, pardaxin, and protegrins, whereas magainin 2, lacticin Q, and melittin are representative peptides of the toroidal pore model [16]. In the carpet model, the peptides, including cecropin, indolicidin and LL-37 [16], adhere to the bacterial membrane, resulting in its disruption [14, 15]. In addition, there are cell-penetrating peptides that show antimicrobial activity by passing through the membrane and targeting intracellular components, and these include buforin 2 as a representative example [12, 19].

These AMPs can also be utilized to develop more potent peptides according to their characteristic mechanisms. Papiliocin, a 37-residue AMP derived from swallowtail butterfly larvae, is a cecropin peptide with low toxicity and potent antimicrobial activity that is mainly conferred by the Trp2 and Phe5 residues [17, 20]. However, the high number of residues constituting papiliocin makes it difficult to synthesize for large-scale production and widespread use. To resolve this limitation, a recent study confirmed that only the 12 residues of the N-terminus of papiliocin (Pap12 peptides) are sufficient for its full antimicrobial activity [21]. We recently reported the development of a 12-mer peptide in which the 12^{th} C-terminal residue of Pap12 peptides was replaced with tryptophan (Trp), designated Pap12-6, which has increased cationicity and hydrophobicity while enhancing the antibacterial action and anti-septic activity by improving the interaction with the bacterial membrane [22]. Valine (Val), a hydrophobic amino acid, is known to make a peptide non-toxic while maintaining its antimicrobial activity [23, 24]. Therefore, in the present study, we designed Pap12-7 with a substitution of Trp with Val at the C-terminus of Pap12-6 as a new peptide with the goal of increasing bacterial cell selectivity while retaining the antimicrobial activity of Pap12-6. We further investigated the detailed antimicrobial mechanisms of Pap12-6 and Pap12-7 under the hypothesis that Pap12-7 would show a distinct mechanism while maintaining the same level of antimicrobial activity as Pap12-6 given the different residue at the terminal position. We examined and compared the antibacterial and anti-inflammatory effects of the two peptides as well as their cytotoxicities and evaluated the mechanism of action via cell membrane effects. Finally, we explored the in vitro and in vivo therapeutic effects of the two peptides by administration to lipopolysaccharide (LPS)-stimulated cells and to a mouse model of sepsis. These findings should lay the foundation for the development of Pap12-7 as a novel antiseptic agent that can overcome therapeutic limitations in the face of antibiotic resistance.

Materials and Methods

Peptide Synthesis

The peptides of interest were synthesized using a standard Fmoc-based, solid-phase synthesis method and purified by reverse-phase high-performance liquid chromatography using a C18 column as described previously [25]. The final purity (>95%) of the peptides was determined using a Vydac C18 column. The molecular weight of the peptides was determined by matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry at Korea Basic Science Institute (KBSI, Ochang, Korea).

Bacterial Strains

Escherichia coli (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 2004), *Acinetobacter baumannii* (KCTC 2508), *Staphylococcus aureus* (KCTC 1621), *Bacillus subtilis* (KCTC 3068), and *Staphylococcus epidermidis* (KCTC 1917) were purchased from the Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology (Taejon, Korea). Clinical isolates of multidrug-resistant *E. coli* (CCARM 1229, CCARM 1238), *P. aeruginosa* (CCARM 2002, CCARM 2003), and *A. baumannii* (CCARM 12010, CCARM 12220) were supplied by the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University in Korea. The *E. coli* K1 strain RS218 (O18:K1:H7) was kindly provided by Dr. Jang-Won Yoon of Kangwon National University (Korea).

Antimicrobial Activity

The minimum inhibitory concentrations (MICs) of the two peptides against the various bacterial strains were assessed using the microdilution method on peptone broth as described previously [25]. In brief, the peptides were added to a bacterial suspension of 2×10^6 CFU/ml in 1% peptone medium and incubated at 37°C for 16 h.

Toxicity against Mammalian Cells

To determine the toxicity of the peptides to sheep red blood cells (sRBCs; Kisanbio, Korea), hemolytic activities were measured and expressed according to a previously described method [25, 26]. The toxicity induced by peptides was also assessed in mouse macrophage RAW 264.7 cells and human keratinocyte HaCaT cells using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay according to standard protocols [25, 26].

Assessment of Inflammatory Cytokines in LPS-Stimulated RAW 264.7 Cells

As previously described, inhibition of inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1α by the peptides in LPS-stimulated RAW 264.7 cells was assessed by enzyme-linked immunosorbent assay (ELISA; R&D Systems,

USA) according to the manufacturer's instructions [27, 28].

Circular Dichroism Experiments

The secondary structures of the peptides were determined by circular dichroism (CD) spectroscopy using a J810 spectropolarimeter (Jasco, Japan). To observe the changes in peptide structure according to the membrane environment, the peptides were treated with 100 mM sodium dodecyl sulfate (SDS) and 50 mM dodecyl phosphocholine (DPC), respectively [22]. The CD spectra were measured from 190 nm to 250 nm at intervals of 0.1 nm at 25°C. The CD data display the average residual ellipticity (θ) in units of deg·cm^2/dmol.

Calcein Dye Leakage Assay

Bacteria and mammalian membrane models were prepared to determine if the peptides selectively act on the bacteria. Large unilamellar vesicles (LUVs) were prepared and composed of egg yolk phosphatidylcholine (PC)/egg yolk phosphatidylglycerol (PG) (7:3, w/w) to mimic Gram-negative membrane and PG/cardiolipin (CL) (6:4, w/w) to mimic Gram-positive membrane as described previously [21, 27]. Mammalian membrane-mimicking LUVs were constructed using PC/cholesterol (CH) (10:1, w/w) [20, 22]. The calcein released from the LUVs was measured at an excitation wavelength of 490 nm and an emission wavelength of 520 nm using an RF-5301PC spectrofluorophotometer (Shimadzu, Japan). The calcein was completely released using 1% Triton X-100 and the percentages were calculated as previously described [21].

Membrane Depolarization

The effects of the peptides on membrane potential changes were measured using 3,3′-dipropythiadicarbocyanine iodide (DiSC3 (5)). Triton X-100 (1 μl, demonstrating 1% peptide-induced membrane depolarization in *E. coli* cells) was measured as described previously [28]. To determine the ability of the peptides to penetrate the outer membrane of Gram-negative bacteria, the leakage of *N*-phenyl-1-napthylamine (NPN), a fluorescent dye, in the outer membrane of *E. coli* KTCT 1682 was measured until the fluorescence no longer increased, as reported previously [28]. Peptide-induced inner membrane permeability was measured using o-nitrophenyl-β-D-galactopyranoside (ONPG) [21]. Absorbance was measured at 420 nm with and without (control) bacteria.

Confocal Microscopy

E. coli (KCTC 1682) grown to the mid-log phage was washed with 10 mM phosphate-buffered saline (PBS) and diluted to reach an optical density of 0.3 at 600 nm. The cells were then treated with fluorescein isothiocyanate (FITC)-labeled peptides at 1× the MIC for 1 h. The cells were washed three times with 10 mM PBS and observed under a confocal laser-scanning microscope (LSM 800; Carl Zeiss, Germany).

Establishment of the Sepsis Mouse Model

BALB/c mice were used to induce sepsis by infection with *E. coli* K1 to evaluate the therapeutic efficacy of Pap12-7. The mice were purchased from Orient (Korea) and maintained under specific pathogen-free conditions for one week before the experiment in a humidity- and temperature-controlled environment. All procedures involved in animal testing were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University, South Korea (IACUC number: KU18163-1).

The BALB/c mice were divided into four groups of five mice/group: control group, injected with PBS into the abdominal cavity; sepsis model group, injected with 0.2 ml of *E. coli* K1 (1×10^7 CFU/mouse); Pap12-7 group, injected with 10 mg/kg Pap12-7; and Pap12-7 sepsis group, injected with 10 mg/kg Pap12-7 and then with *E. coli* K1 (1×10^7 CFU/mouse) after 1 h. The survival rate was assessed in the four groups for up to 4 days (0, 6, 12, 18, 24, 30, 36, 48, 60, 72, 84, 96 h).

Analysis of the sepsis model was conducted as described previously [22]. The amount of TNF-α and IL-6 in the serum and lung lysate of the mice was measured using sandwich ELISA kits. Aspartate aminotransferase (AST), alanine amino transferase (ALT), and blood urea nitrogen (BUN) levels in the serum were measured using the standard kit from Asan Pharmaceutical as described previously [29]. The lungs were extracted and fixed with 4% paraformaldehyde solution, stained with hematoxylin and eosin, and observed under a microscope.

Statistical Analysis

Measurements were taken at least three times and statistical analyses were carried out using Graphpad Prism software. Dunnett's multiple comparisons test (Prism 7.0, Graphpad Software Inc., USA) was used for comparisons of multiple groups. Values were considered statistically significant at $p < 0.05$.

Results

Peptide Characteristics

Papiliocin is known as an α-helical AMP and contains 37 amino acids with potent antimicrobial activity. In order to develop short peptide antibiotics, previous studies have shown that the 12-meric peptide of papiliocin maintains its amphipathic structure while maintaining its antimicrobial activity. Pap12-6 was designed to increase both cationicity and the interaction between hydrophobicity and membrane by replacing the last residue with Trp. In this study, we designed Pap12-7 as a novel peptide antibiotic by substituting Val for Trp12 at the C-terminus of Pap12-6 with an aim to increase bacterial cell selectivity while maintaining the antimicrobial activity. As shown in Table 1, both peptides have a +6 charge, whereas the hydrophobicity and amphipathic moment of Pap12-7 were slightly decreased compared to those of Pap12-6.

Table 1. Peptide designs and their key physicochemical properties.

Peptide	Sequence	Charge	Mean hydrophobicity[a] <H>	Mean amphipathic moment[b] <μH>	MW
Pap12-6	RWKIFKKVVKKW-NH2	+6	0.381	0.736	1645
Pap12-7	RWKIFKKVVKKV-NH2	+6	0.295	0.653	1559
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2	+5	0.511	0.394	2847

The mean hydrophobic moment[a] <H> or mean amphipathic moment[b] <mH> were calculated using HeliQuest (available at: http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py).

Antibacterial Activity

The antimicrobial activities of Pap12-6 and Pap12-7 peptides were tested against three standard Gram-negative bacteria (*E. coli*, *P. aeruginosa and A. baumannii*) three standard gram-positive bacteria (*S. aureus*, *B. subtilis*, and *S. epidermidis*), and nine MDR bacteria (MDREC CCARM 1229, MDREC CCARM 1238, MDRPA CCARM 2002, MDRPA CCARM 2003, MDRAB CCARM 12010, MDRAB CCARM 12220, MRSA CCARM 3090, MRSA CCARM 3114, and MRSA CCARM 3126) using microbroth dilution method. Table 2 shows that Pap12-6 and Pap12-7 exerted the same level of antibacterial activity against all strains tested. Both the peptides showed approximately 2-fold higher activity against Gram-negative bacteria than against Gram-positive bacteria.

Table 2. Antibacterial activities of Pap12 peptides against standard and drug-resistant bacterial strains.

Microorganisms	Minimal inhibitory concentration (MIC) (μM)		
	Pap12-6	Pap12-7	Melittin
Gram negative			
E. coli	4	4	4
A. baumannii	2	2	2
P. aeruginosa	4	4	4
MDREC 1229	8	8	4
MDREC 1238	8	8	8
MDRPA 2002	8	8	8
MDRPA 2003	8	8	4
MDRAB 12010	8	8	4
MDRAB 12220	8	8	4
GM[a]	6.44	6.44	4.66
HC_5	400	800	2.3
Therapeutic index[b]	62	124	0.49
Gram positive			
S. aureus	4	4	4
B. subtilis	8	8	2
S. epidermidis	8	8	2
MRSA 3090	16	16	8
MRSA 3114	16	16	8
MRSA 3126	16	16	8
GM[a]	11.33	11.33	5.33
HC_5	400	800	2.3
Therapeutic index[b]	35.3	70.6	0.43

GM[a] is the geometric mean of the minimum inhibitory concentrations (MICs) against all bacteria.

Therapeutic index[b] = HC_5/MIC, where HC_5 is the peptide concentration resulting in 5% hemolysis of fresh sheep erythrocytes. The therapeutic index was calculated by doubling the maximum concentration at which hemolytic activity was not observed. The greater the therapeutic index, the larger the cell selectivity.

Toxicity against Mammalian Cells

The toxicity of Pap12-6 and Pap12-7 to sRBCs is shown in Fig. 1A. Pap12-6 showed only 6.2% hemolysis at 400 μM while Pap12-7 did not show hemolysis. Pap12-6 resulted in 22.1% reduction in the survival or Raw 264.7 cells at 50 μM but had no toxicity to HaCaT cells, whereas Pap12-7 showed no toxicity in either mammalian cell line. These results implied that Pap12-7 has extremely high bacterial cell selectivity and Pap12-6 also showed low cytotoxicity against mammalian cells (Figs. 1B and 1C). The positive control peptide, melittin, was cytotoxic even at even very low concentrations.

Inflammatory Cytokines in LPS-Stimulated RAW 264.7 Cells

The inhibition of the production of inflammatory cytokines in LPS-simulated RAW 264.7 was measured using an ELISA. Both peptides significantly inhibited the production of TNF-α, IL-1α, and IL-6 (Fig. 1D). Pap12-6 inhibited TNF-α by 70.8% and 74.9% at 10 uM and 20 uM and Pap12-7 inhibited TNF-α by 62.6% and 66.1% at 10 uM and 20 uM, respectively. Pap12-6 showed 49.1% and 75.5% inhibition of inhibition of IL-1α production at 10 uM and 20 uM, respectively, while Pap12-7 showed 47.6% and 71.6% inhibition of IL-1α production at 10 uM and 20 uM, respectively. Pap12-6 inhibited IL-6 by 76.4% and 99.5 at

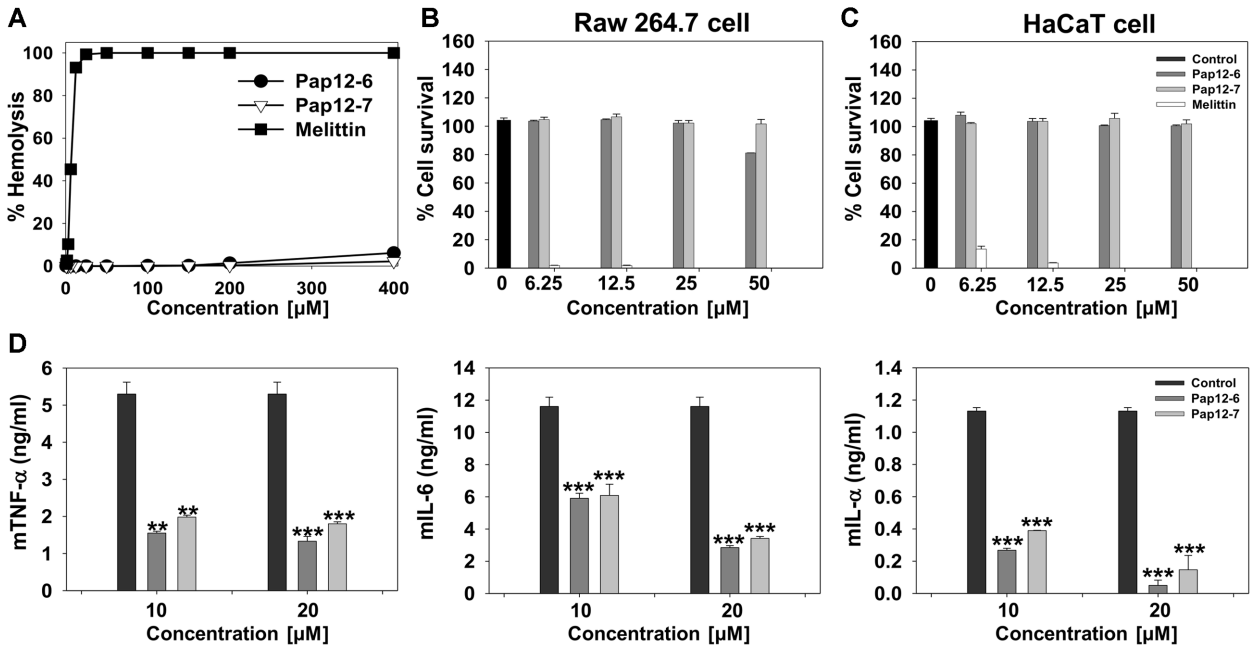

Fig. 1. Toxicities and anti-inflammatory activities of Pap12 peptides.
(**A**) Hemolysis in sheep red blood cells. (**B**) Toxicity against mouse macrophage RAW 264.7 cells and (**C**) human keratinocyte HaCaT cells. (**D**) Inhibition of mouse TNF-α by peptides (10 μM and 20 μM) in LPS (50 ng/ml)-stimulated RAW 264.7 cells. Levels of mouse IL-6 and IL-1α in the supernatant of LPS (50 ng/ml)-stimulated RAW 264.7 cells exposed to the peptides for 16 h. Error bars represent ± standard error of the mean. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n.s. no statistical significance).

10 uM and 20 uM, respectively while Pap12-7 inhibited IL-6 by 65.6% and 87.1% at 10 uM and 20 uM, respectively. These results implied Pap12-7 retained the anti-inflammatory activity of Pap12-6.

CD Experiments

Pap12-6 and Pap12-7 both showed a random coil structure in aqueous solution with similar contents of α-helix structures in the presence of SDS and DPC micelles (Fig. 2A), implying that the amphiphilic α-helical structure plays an important role in the high antimicrobial activity.

Calcein Dye Leakage Assay

Bacterial membrane consists mainly of a mixture of zwitterionic and anionic phospholipids such as phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) and cardiolipin (CL) [30–34]. In contrast, mammalian membrane is composed mainly of PC and cholesterol (CH) [32–35, 39]. The composition of the lipid membrane of Gram-negative bacteria consists of zwitterionic PE (about 75% of membrane lipids), anionic lipids PG (about 20%) and CL (5%) [32, 33, 35–37]. Gram-positive bacterial lipid membranes consist mainly of PG and CL [33–35, 38]. For example, *S. aureus*, a representative Gram-positive bacterium, consists of 58% PG and 42% CL [38]. Therefore, we conducted a calcein dye leakage experiment using artificial liposomes to investigate the interaction between membrane and peptide. PC/PG (7:3, w:w) LUVs were used to mimic Gram-negative bacteria membranes [22, 33, 40] while PG/CL (6:4, w:w) LUVs were used to mimic Gram-positive bacterial membranes [27, 33, 41]. PC/CH (10:1, w:w) LUVs were used to mimic mammalian cells [22, 27, 40, 42]. Pap12-7 showed lower permeability than Pap12-6 against PC/PG (7:3, w/w) LUVs, PG/CL (6:4, w/w) LUVs, and PC/CH (10:1, w/w) LUVs (Fig. 2B). These results indicated that Pap12-7 might have a different antibacterial mechanism from that of Pap12-6. In contrast, melittin, used as a positive control, showed strong permeation in all membrane models.

Membrane Depolarization

Membrane depolarization capacity was measured to further investigate the antibacterial mechanisms of Pap12-6 and Pap12-7 based on the release of membrane potential-sensitive dyes from bacteria (Fig. 3C). The extent to which peptides depolarize the membranes of Gram-negative

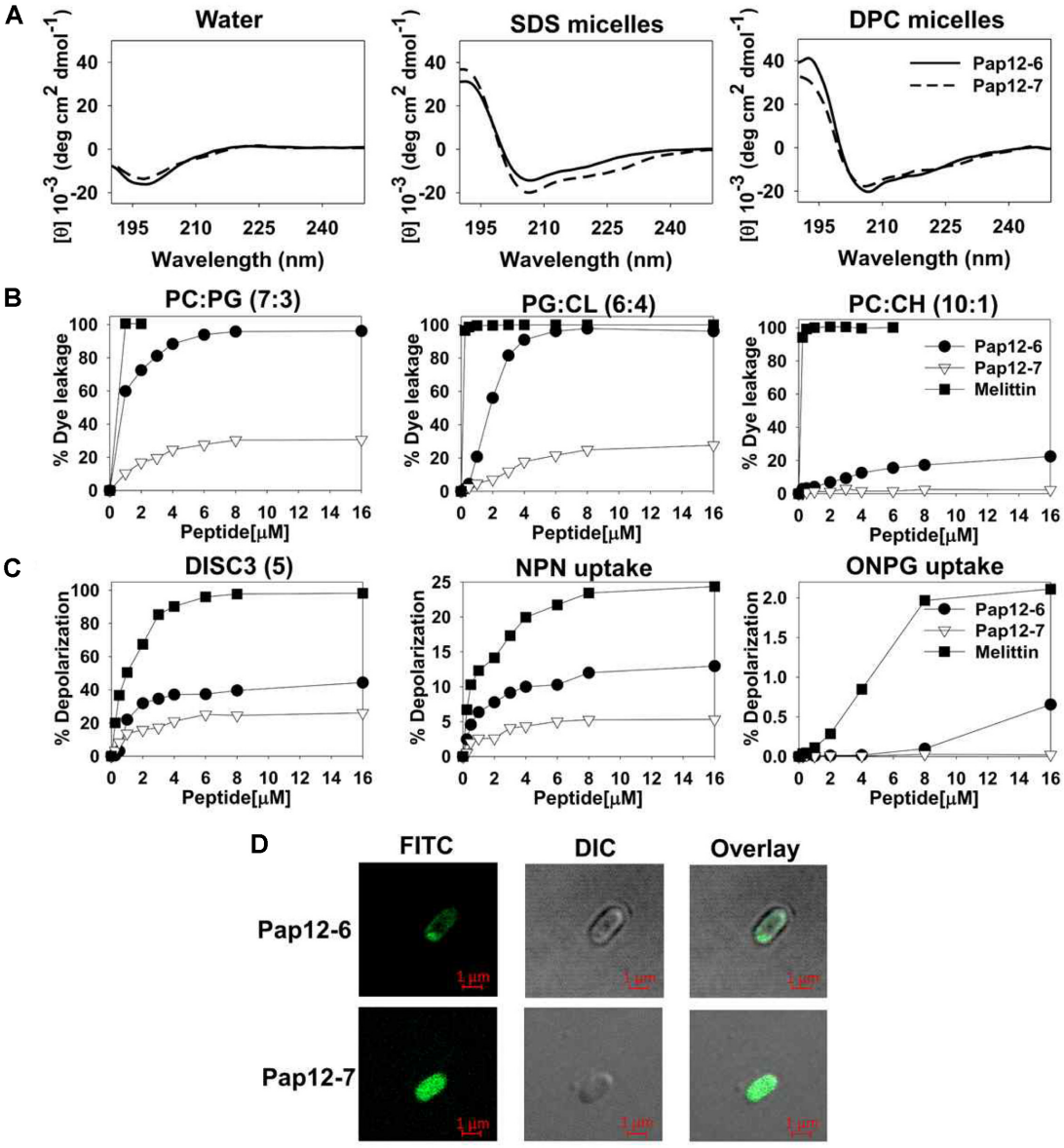

Fig. 2. Biophysical tests for peptide–membrane interactions.

(**A**) Circular dichroism spectra for 50 μM Pap12-6 and Pap12-7 peptides in aqueous solution, 100 mM SDS micelles, and 50 mM DPC micelles. (**B**) Peptide-induced dye leakage from LUVs mimicking the Gram-negative bacterial membrane (PC:PG = 7:3), LUVs mimicking the Gram-positive bacterial membrane (PG:CL = 6:4), and LUVs mimicking the mammalian membrane (PC:CH = 10:1). (C) Peptide-induced membrane depolarization measured with DiSC3(5) dye against intact *E. coli*, outer membrane permeability assessed by NPN assays on *E. coli* KCTC 1682 cells, and inner membrane permeability assessed by ONPG assays on *E. coli* ML35p cells. (**D**) *E. coli* treated with Pap12-6 or Pap12-7 labeled with FITC for 1 h.

bacteria and their effects on the outer and inner membranes were examined. In the Gram-negative bacterium *E. coli*, Pap12-7 showed about 18% reduced depolarization than did Pap12-6. In addition, Pap12-7 showed reduced NPN uptake compared to Pap12-6, indicating a more intact outer membrane. Similarly, Pap12-7 showed almost no permeability of the bacterial endothelium based on the chromophore yielded from cytoplasmic ONPG, whereas Pap12-6 showed uptake on the membrane. These results indicate that two peptides with similar antimicrobial activity have different antimicrobial mechanisms. Pap12-6 exhibits antimicrobial activity by depolarizing the

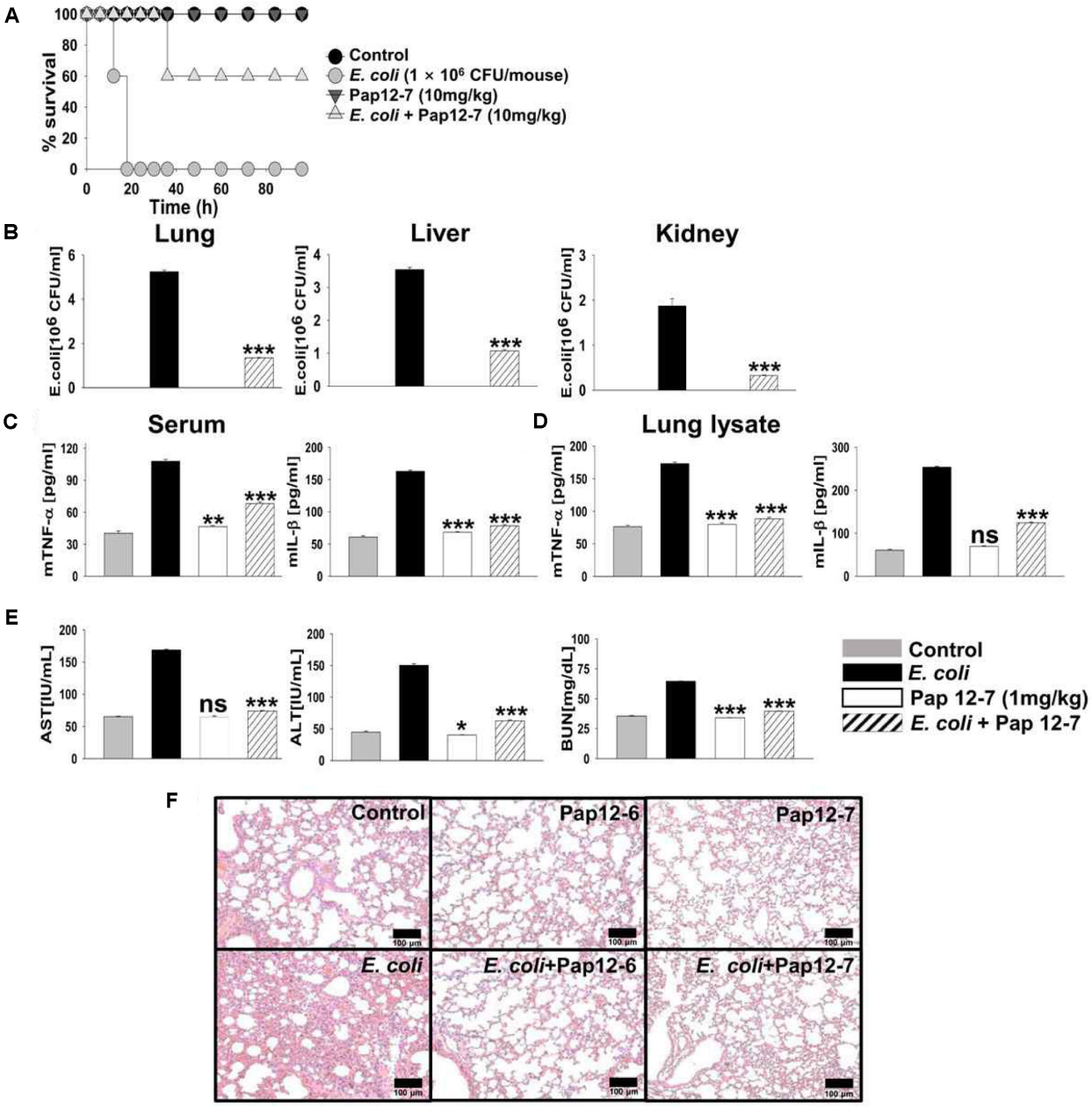

Fig. 3. Sepsis inhibition effect of Pap12-7 in an *E. coli*-induced sepsis mouse model.
(**A**) Survival rate of *E. coli*-induced sepsis mouse model treated with or without Pap12-7. (**B**) Inhibition of bacterial growth in the lung, liver, and kidney of the *E. coli*-induced sepsis model. (**C**) Inhibition of cytokine production (mTNF-α and mIL-6) in serum. (**D**) Inhibition of cytokine production (mTNF-α and mIL-6) in lung lysates. (**E**) AST, ALT, and BUN levels in *E. coli* induced sepsis mouse model. Four different treatment groups in E are denoted in gray (PBS), black (1×10^6 CFU/mouse *E. coli*,), white (1 mg/kg Pap12-7), and diagonal (1 mg/kg Pap12-7 with *E. coli* K1). (**F**) Inhibitory effect of Pap12-6 and Pap12-7 on lung inflammation in a bacterial sepsis mouse model. BALB/c mouse Control, BALB/c mice were treated with 1 mg/kg of Pap12-6 or Pap12-7, BALB/c mice underwent intraperitoneal infection with *E. coli*, BALB/c mice were pretreated with 1 mg/kg of Pap12-6 or Pap12-7, followed by intraperitoneal infection with *E. coli* (1×10^6 CFU/mouse). The error bars represent ± SEM. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n.s. represents no significance).

membrane, while Pap12-7 exhibits antimicrobial activity by other mechanisms without depolarizing the membrane.

Confocal Microscopy

We found that Pap12-6 and Pap12-7 had different degrees

of depolarization of *E. coli* membranes. Therefore, to observe how the two peptides act on the *E. coli* membrane, we visualized these interactions using a confocal microscope. Confocal microscope observations showed that the cells treated with Pap12-6 clearly emitted FITC fluorescence on the cell membrane and Pap12-6 was attached to the outer membrane. In contrast, cells treated with Pap12-7 showed FITC fluorescence inside cells, indicating that Pap12-7 penetrated the membrane (Fig. 3D).

Pap12-7 Prevents Septic Shock in a Mouse Model

The antibacterial and anti-inflammatory actions of Pap12-7 and Pap12-6 have very similar antimicrobial activities in vitro. Their therapeutic potency in vivo can be examined using a septic shock mouse model. In our previous study, we already demonstrated that Pap12-6 has anti-septic activity. In this study, the efficacy of the newly designed peptide Pap12-7 in preventing septicemia was investigated in a mouse model of sepsis induced by *E. coli* K1. All mice in the control group survived for more than 4 days. However, 100% of the mice infected with *E. coli* K1 died after 18 h, whereas mice treated with 10 mg/kg Pap12-7 1 h prior to infection with *E. coli* K1 showed a 100% survival rate up to 20 h and a survival rate of 60% up to 96 h. (Fig. 3A).

To further investigate the effect of Pap12-7 on the prevention of sepsis, the organs of sepsis-induced mice were extracted and further experiments on anti-septicemia were performed. Mice administered Pap12-7 (1 mg/kg) 1 h before infection showed reduction in the number of bacteria in the lung, liver, and kidney by 74.2%, 69.8%, and 82.7%, respectively (Fig. 3B). Moreover, Pap12-7 inhibited the high levels of TNF-α and IL-6 in the serum and lung lysates induced by *E. coli* K1 (Figs. 3C and 3D). Pap12-7 also considerably reduced the AST, ALT, and BUN levels of the serum, indicating that the peptide could effectively reduce the liver and kidney cell damage caused by *E. coli* K1 infection (Fig. 3E).

Histopathological examination of Pap12-6 and Pap12-7 was conducted for the first time to examine the infiltration of polymorphic neutrophils in the infected lung tissues (Fig. 3F). Compared with the *E. coli* K1-treated group, the mice treated with both peptides showed a marked decrease in the infiltration of immune cells into the lung. Treatment with each peptide alone resulted in similar numbers of immune cells to those of the normal lung tissue, confirming that the toxicity was low. In other words, both Pap12-7 and Pap12-6 could prevent sepsis. Therefore, Pap12-7 has an effect of preventing sepsis similar to Pap12-6 and can function as an effective antiseptic peptide with low cytotoxicity.

Discussion

Despite the many advances in the treatment of bacterial infections, antibiotic resistance has become a significant clinical challenge and public health threat, with reduced treatment options available [4–6]. In particular, sepsis is a disease by which bacteria infect the blood, and is associated with acute toxicity [43]. The development and research of newly emerging AMPs offer promise for overcoming this situation. Thus, we are actively seeking to discover and develop new AMPs with low toxicity and high activity [9].

Previously, we reported the development of Pap12-6 from papiliocin with a potent anti-septic activity and antimicrobial effect by targeting the bacterial membrane [22]. In the present study, we designed a new peptide by replacing the last Trp residue of Pap12-6 with Val (Pap12-7) to increase bacterial selectivity as well as different antibacterial mechanisms. To investigate the mechanism of action of the two peptides, we performed a calcein dye leakage assay and membrane depolarization experiments, which showed distinct membrane interactions of the two peptides despite only one amino acid difference at the C-terminus. Pap12-6 showed very high dye leakage against LUVs mimicking the bacterial membrane, while Pap12-7 showed significantly lower dye leakage. Both peptides had minimal effects on LUVs mimicking the mammalian membrane. Membrane depolarization experiments further confirmed that Pap12-7 resulted in significantly lower depolarization compared to Pap12-6 against *E. coli*. This is evidence that Pap12-7 exerts antimicrobial activity without depolarizing the membrane. In addition, confocal microscopy confirmed that Pap12-6 is attached to the membrane of *E. coli* while Pap12-7 is located inside the *E. coli* cell, indicating its ability to penetrate the membrane.

Pap12-6 and Pap12-7 have the same amino acid sequence except for the last residue at C-terminus but have different antibacterial mechanisms. Therefore, Trp or Val residues at the C-terminus of amphipathic-helical Pap12 peptides affect their mechanism of action. Leu-rich peptides are known to depolarize bacterial cells to a greater extent than Val-rich peptides, and the substitution of Val for Leu could further reduce the cytotoxicity [23]. In addition, it has been reported that cell-penetrating peptide was designed to consist of Arg and Val only and did not show cytotoxicity [44]. Trp is frequently found in membrane proteins,

preferentially localized near the membrane–water interface, and is considered to play an important role in anchoring the protein to the phospholipid bilayer. Since Pap12-6 has two Trp residues at the N-terminus and C-terminus, the indole rings of these two Trps may anchor to the phospholipid head group. These interactions may result in the alpha-helix resting on the surface of the membrane, which would induce membrane lysis [45–47]. In contrast, Val only has a hydrophobic aliphatic side chain, which can form a stable interaction with the hydrophobic acyl chain of phospholipids and may result in penetrating the bacterial cell membrane. Consequently, Pap12-7 with Val at the C-terminus showed increased bacterial cell selectivity but retained antibacterial activity against all bacteria tested, despite a different antibacterial mechanism compared to Pap12-6. Collectively, we concluded that Pap12-7 showed its antimicrobial activity by penetrating the bacterial membrane. However, the precise mechanisms of Pap12-7 should be investigated further.

Both peptides also exhibited similar inhibitory effects on production of TNF-α, IL-1α, and IL-6 in RAW 264.7 cells induced by LPS. Similar to Pap12-6 as reported previously, Pap12-7 also showed good efficacy in sepsis control experiments in mice by effectively reducing the growth of *E. coli* in vivo, and inhibiting the production of pro-inflammatory cytokines TNF-α and IL-β in the blood and lung lysates of infected mice. In addition, this study showed for the first time that both peptides protected against excessive neutrophil infiltration in the infected mouse lung tissues, confirming that both peptides significantly suppressed the inflammation in the lung induced by Gram-negative bacteria infection. Therefore, the new peptide Pap12-7 has potent antibacterial and anti-inflammatory activities in vitro and in vivo with high bacterial cell selectivity, demonstrating its potential as a strong antiseptic agent.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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