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Novel chimeric peptide with enhanced cell specificity and anti-inflammatory activity

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ABSTRACT

An antimicrobial peptide (AMP), Hn-Mc, was designed by combining the N-terminus of HPA3NT3 and the C-terminus of melittin. This chimeric AMP exhibited potent antibacterial activity with low minimal inhibitory concentrations (MICs), ranging from 1 to 2 μM against four drug-susceptible bacteria and ten drug-resistant bacteria. Moreover, the hemolysis and cytotoxicity was reduced significantly compared to those of the parent peptides, highlighting its high cell selectivity. The morphological changes in the giant unilamellar vesicles and bacterial cell surfaces caused by the Hn-Mc peptide suggested that it killed the microbial cells by damaging the membrane envelope. An *in vivo* study also demonstrated the antibacterial activity of the Hn-Mc peptide in a mouse model infected with drug-resistant bacteria. In addition, the chimeric peptide inhibited the expression of lipopolysaccharide (LPS)-induced cytokines in RAW 264.7 cells by preventing the interaction between LPS and Toll-like receptors. These results suggest that this chimeric peptide is an antimicrobial and anti-inflammatory candidate as a pharmaceutic agent.

1. Introduction

The emergence of drug-resistant pathogens is due to the abuse and misuse of antibiotics in the biomedical fields. Drug-resistance is induced mostly by two mechanisms, inhibiting drug accumulation into the cytosol via multidrug efflux pumps [1–3] and phenotypic changes during infection, such as biofilms [4,5]. Until now, a number of studies have searched for new antibiotics with a different mechanism. Among them, antimicrobial peptides (AMPs), which are host defense molecules in a broad range of organisms, is a promising new antimicrobial candidate [6–8]. Although the sequences of AMPs vary, they have similar characters, such as a broad-spectrum of antimicrobial activity, amphipathicity, small length, cationic net charge, and rapid killing kinetics [9–13]. Their cellular targets are the plasma membrane and cytosolic components, but their mode of action in microbes is not completely understood [14,15]. Membranolytic AMPs induce alterations of the membrane potential or direct membrane disruption via "barrel-stave", "carpet", "toroidal", and "aggregation" models [16–21]. On the other hand, some AMPs are translocated to the cytoplasm of microbial cells via spontaneous lipid-assisted and receptor or channel-mediated translocation [15]. The precise mode of action in which AMPs enter spontaneously across the microbial membranes has not been determined. On the other hand, several models, such as the transient pore formation [22], lipid phase boundary defects [23] and the disordered toroidal pore formation [24,25], have been proposed. The internalized peptides inhibit or kill bacterial cells by inhibiting macromolecules synthesis, chaperone-mediated protein folding, cell wall synthesis, and cytoplasmic membrane septum formation. On the other hand, the uptake mechanism and cytoplasmic targets of many bacteria-penetrating peptides have not been demonstrated or yet undefined.

In the present study, the N-terminus of HPA3NT3 and C-terminus of melittin were collected to design a novel antimicrobial peptide with high antimicrobial activity and low cytotoxicity. HPA3NT3 is an analog peptide, derived from HP(2–20) with residues 2–20 of the parental HP from the *Helicobacter pylori* ribosomal protein L1. The peptide has potent antimicrobial activity through

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membranolytic action [26–28]. However, it is difficult to apply *in vivo* due to the cytotoxicity and aggregation at high concentrations. Melittin, from the European honeybee venom (*Apis mellifera*), is cytotoxic peptide with 26 amino acids and possesses a broad spectrum of antimicrobial activity [29,30]. Although its cytotoxicity and allergenicity are high, a number of studies have examined potential applications in therapeutic and biotechnological fields, focusing on bacterial infections [31], rheumatoid arthritis [32], arteriosclerosis [33], immunologic adjuvants [32], cancer [34], and endosomolytic material in drug delivery systems [35,36]. The substitution and deletion of amino acids are general methods to reduce the cytotoxic effect, but it is sometimes limited due to the length and structure of the peptide. Hybridization is a useful way that can combine the advantages of other peptides. In particular, a hybrid undecapeptide from cecropin A and melittin possess potent antimicrobial activity while displaying low cytotoxicity [37]. An improved version of this hybrid peptide has been developed with retro and retroenantio analogs [38,39].

In this study, a chimeric peptide, Hn-Mc (FKRLKKLISWIKRKRQQ-NH_2), was designed to reduce the cytotoxic effects and have both antimicrobial and anti-inflammatory activities. The *in vivo* antibacterial activity of the peptide was assessed in mouse model infected by drug-resistant *Pseudomonas areuginosa*.

2. Materials and methods

2.1. Materials

Phosphatidylethanolamine (PE, from *Escherichia coli*), rhodamine-PE, and phosphatidylglycerol (PG, from *E. coli*) were from Avanti Polar Lipids (Alabaster, AL). Fluorescein isothiocyanate (FITC)-labeled lipopolysaccharide (LPS), *E. coli* O111:B4 LPS and ciprofloxacin were

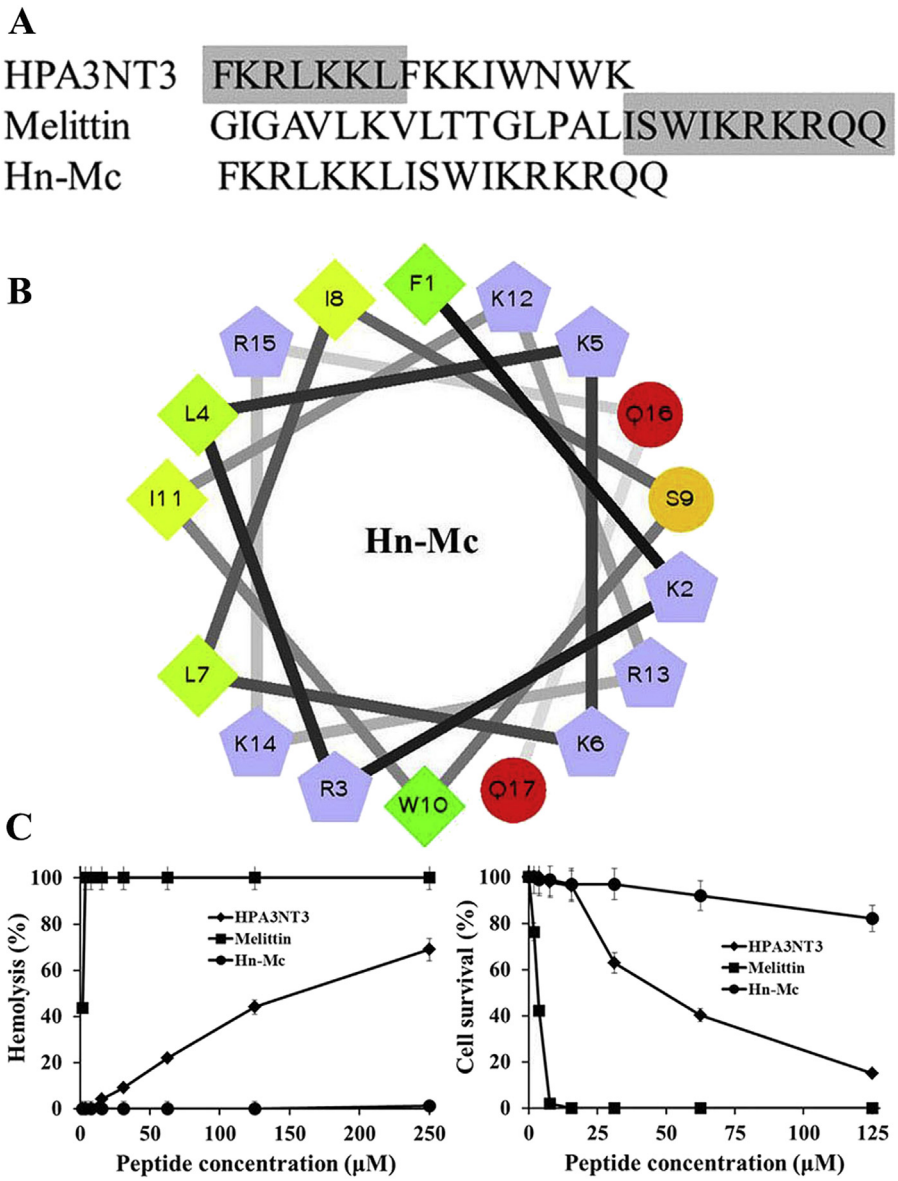

Fig. 1. Design (A), helical wheel projection (B), and cytotoxic effects (C) of the chimeric antimicrobial peptide, Hn-Mc. Hn-Mc peptide was hybridized by N-terminus of HPA3NT3 (1–7) and C-terminus of melittin (17–26) (A). The helical wheel projection was performed using online program of the Helical Wheel Projections: http://rzlab.ucr.edu/scripts/wheel/wheel.cgi (B). Synthetic peptides were evaluated for hemolysis (left panel) and cytotoxicity (right panel) in rat red blood cells and HaCaT cells, respectively (C).

Table 1
MICs of peptides against drug-susceptible and drug-resistant bacteria.

Bacteria	MIC (μM)				
	HPA3NT3	Melittin	Hn-Mc	Ciprofloxacin	Gentamicin
Drug-susceptible bacteria					
E. coli	2	2	1	–	–
P. aeruginosa	2	2	2	0.5	0.5
S. aureus	4	2	2	1	0.5
B. subtilis	2	2	2	–	–
Drug-resistant bacteria					
P. aeruginosa DRPA-001	2	16	1	2	>64
P. aeruginosa DRPA-002	2	8	1	>64	8
P. aeruginosa DRPA-003	2	16	1	>64	>64
P. aeruginosa DRPA-004	1	16	1	16	1
P. aeruginosa DRPA-005	2	8	2	0.5	8
S. aureus DRSA-001	2	2	2	>64	0.5
S. aureus DRSA-002	2	2	2	>64	>64
S. aureus DRSA-003	2	2	1	>64	>64
S. aureus DRSA-004	2	2	2	1	>64
S. aureus DRSA-005	2	2	2	>64	>64

obtained from Sigma-Aldrich Co. 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from CEM Co. All other reagents were of analytical grade.

2.2. Peptide synthesis

All peptides were prepared using solid-phase methods with Fmoc-protected amino acids on a Liberty Microwave Peptide synthesizer (CEM Co., Matthews, NC). Rink amide 4-methylbenzhydry lamine resin (Novabiochem) (0.55 mmol/g) was used to create the amidated peptides. The peptides were cleaved from the resins, followed by precipitation and extraction with ether. After synthesis of the peptides, the crude peptides were purified on a Zorbax C18 column (21.2 × 250 mm, 300 Å, 7-μm) on a Waters preparative HPLC system, using 0–60% acetonitrile gradient in water with 0.1% trifluoroacetic acid. The purity of the isolated peptide was determined on a Shimadzu Analytical HPLC system. The molecular masses of the peptides were confirmed using a matrix-assisted laser desorption ionization mass spectrometer (MALDI II, Kratos Analytical Ins.) [40].

2.3. Antibacterial assay

The antibacterial activity was examined by a microdilution assay using *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15692) and *Staphylococcus aureus* (ATCC 25923), which were purchased from the American Type Culture Collection, and *Bacillus subtilis* (KCTC 1998) from the Korean Collection for Type Cultures. Drug-resistant *P. aeruginosa* (DRPA-001~005) and *S. aureus* (DRSA-001~005) were strains isolated from patients in a hospital. The assay was followed by a previous report [40].

2.4. Hemolysis and cytotoxicity

The hemolytic activity against fresh rat red blood cells (rRBCs) and the cytotoxic activity against HaCaT (human keratinocyte) cells were examined using the method described previously [27,40].

2.5. Preparation and visualization of giant unilamellar vesicles (GUVs)

Rhodamine-PE/PE/PG (0.1/6.9/3, w/w/w) mixtures were prepared in chloroform-methanol (9/1, v/v). Two hundred μL of a lipid mixture were deposited onto indium tin oxide (ITO)-coated glass slides (Sigma–Aldrich, St. Louis, MO), which were then spin-coated at 500 rpm for 5 min. The residual solvents were removed by evacuation in a vacuum for 4 hr. To form an electroformation chamber (25 × 25 × 1 mm), two ITO glass slides (one with a lipid film) were separated by a poly(dimethylsiloxane) spacer. The chamber was then filled with 5 mM HEPES buffer containing 0.1 M sucrose through a hole in the poly(dimethylsiloxane) spacer. A 1.7 V (peak to peak), 10 Hz AC field was then applied immediately to the ITO slides using a function generator (Agilent 33220A, Agilent Technology, US). After 2 h, the electric field was changed to 4 V, 4 Hz for 10 min to detach the liposomes that formed on the slides. The liposome solution was removed gently from the electroformation chamber, and The solutions were diluted in 5 mM HEPES buffer containing 0.1 M glucose. Aliquots of the resulting GUV suspension were then deposited on a microscope slide and allowed to settle for 1 min. The liposomes were then examined by inverted fluorescence phase contrast microscope (IX71, Olympus, Tokyo, Japan). The images were recorded using a digital CCD camera (DP71, Olympus) and video recorder and analyzed using the software provided by the manufacturer [40].

2.6. Scanning electron microscopy (SEM)

Midgrowth-phase *E. coli* cells were suspended at a 10^8 colony forming unit (CFU)/mL in phosphate buffered saline buffer (pH 7.4) and then incubated at 37 °C with Hn-Mc (2 μM). The controls were run in the absence of a peptide solution. After 1 h, the cells were fixed with an equal volume of 2.5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). After 2 h fixation at 4 °C, the samples were washed extensively with 0.1 M cacodylate buffer (pH 7.4). The supernatant filters were then treated with 1% (w/v) osmium tetroxide, washed in 5% (w/v) sucrose in cacodylate buffer, and then dehydrated with a graded series of ethanol. After lyophilization and gold coating, the samples were observed by SEM (JSM-7100F, JEOL, Tokyo, Japan) [27].

2.7. NO and TNF-α production by LPS-stimulation in RAW 264.7 cells

The murine macrophage cell line, Raw 264.7, was grown on RPMI-1640 supplemented with 10% fetal bovine serum and plated at a density of 1×10^6 cells/mL in a 96-well microplate. After incubation overnight, the cells were stimulated with LPS (*E. coli* 0111:B4) at a final concentration 1 μg/mL. Twenty-four hours after

adding the peptides, the nitrite and TNF-α levels in the culture supernatants were analyzed using a Griess reagent and Quantikine murine kits from R&D Systems (Minneapolis, MN), respectively.

2.8. Effect of peptide on LPS binding to macrophages

The FITC-labeled LPS was incubated with the Hn-Mc peptide at the indicated concentrations for 10 min at room temperature (RT), after which the mixtures were added to the RAW 264.7 cells (2×10^5 cells/well) cells for 12 h. To remove the excess LPS, the cells were washed with PBS (pH 7.4). The binding, localization and penetration of FITC-LPS in the presence of Hn-Mc was observed by fluorescence microscope.

2.9. In vivo experiment

In vivo studies were performed in accordance with the National Institutes of Health guidelines for the ethical treatment of animals, and all animal studies were approved by the Animal Care Committee of Sunchon National University (SCNU_IACUC-2013-7, 1 July 2013). Five-week-old ICR mice were injected intraperitoneally with drug-resistant *P. aeruginosa* DRPA-003 (1×10^8 CFU/mL), followed by an intraperitoneal injection of the samples after 1 h. The survival rate was recorded over a 10-day period. The lungs from the sacrificed mice were removed and washed with PBS, after which they were fixed in 4% paraformaldehyde for 12 h and dehydrated 3 times with 50% to 100% ethanol for 2 h. Subsequently, xylene was substituted for 1 h, and the paraffin-embedded samples were cut to 5 μm pieces (Microtome, Thermo-scientific, Waltham, MA, USA) and stained with hematoxylin and eosin staining (H&E). The histology of samples was observed by fluorescence microscopy (IX71, Olympus, Tokyo, Japan) [41].

2.10. Statistical analysis

The mean values of at least four independent determinations ± SD and the *P*-values (Student *t*-test) were calculated using Excel software.

3. Results and discussion

3.1. Designation and antibacterial activity of peptides

To design a novel model AMP, the N-terminus of HPA3NT3 (1–7) [27] and the C-terminus of melittin (17–26) were combined as a chimera form (Hn-Mc, Fig. 1A). The hydrophobic and hydrophilic amino acids were distributed evenly in the sequence of Hn-Mc and its amphipathic peptide (Fig. 1B). As shown in Table 1, Hn-Mc exerts potent antibacterial activity against a broad spectrum of pathogenic strains containing the drug-susceptible and drug-resistant bacteria within 1~2 μM. The results may be due to the decrease in mean hydrophobicity and increase in the net charge of the Hn-Mc peptide, compared to the parent peptides. In particular, hydrophobicity is an important factor in cytotoxicity. HPA3NT3 and melittin revealed the hemolysis of 68.2% and 100% at 250 μM, respectively, but Hn-Mc was 1.1% at the same concentration (Fig. 1C-left). The IC_{50} of HPA3NT3, melittin and Hn-Mc against HaCaT cells were 49.5, 2.8 and 357.5 μM, respectively (Fig. 1C-right). This suggests that Hn-Mc is an excellent model peptide with potent antibacterial activity and non-cytotoxicity.

3.2. Hn-Mc exhibits membranolytic action

The morphological changes of artificial GUV (~40 μm), bacterial mimic vesicle composed of Rho-PE/PE/PG (0.1/6.9/3, w/w/w) were observed to investigate the mode of action of Hn-Mc. As soon as the

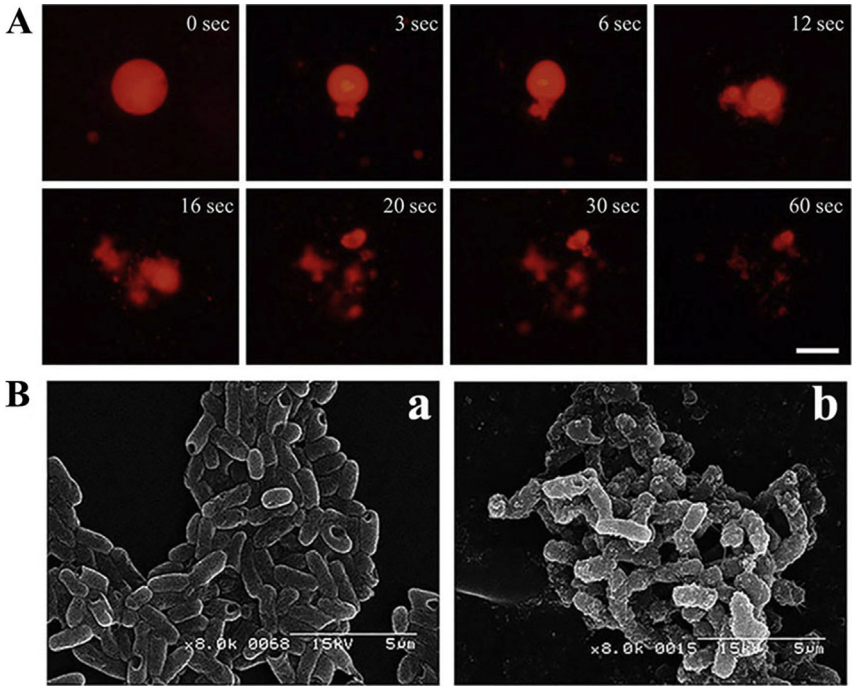

Fig. 2. Memranolytic effects of Hn-Mc peptide in giant unilamellar vesicle (GUV) (A) and bacterial cells (B). The Hn-Mc peptide was subjected at 2 μM in GUV, composed of Rho-PE/PE/PG lipid (0.1:6.9:3, w/w), and was recorded digitally in time-dependent manner. Scale bar is 25 μm (A). *E. coli* cells without (a) or with 2 μM of Hn-Mc (b) was observed by using scanning electron microscopy (SEM).

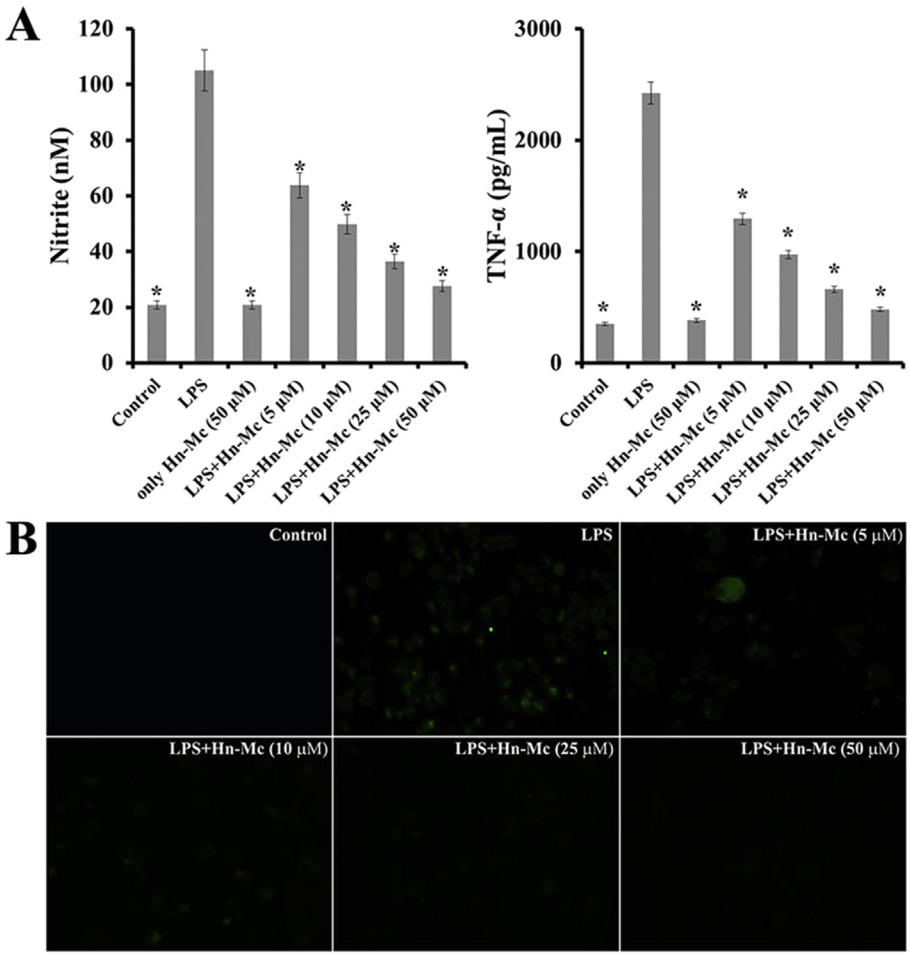

Fig. 3. Anti-inflammatory effects of Hn-Mc peptide in Raw 264.7 cells. Secreted nitric oxide and TNF-α was measured from the supernatants after 24 hr of LPS-stimulation with peptides. (*P < 0.01) (A). FITC-labeled LPS and peptides were incubated for 12 hr in Raw 264.7 cells, washed with PBS and observed by fluorescence microscopy (B).

peptide was added to the GUV solution, the shape of the spherical GUV was changed severely to that of a water balloon. A nick in GUV appeared, and small vesicles were budded into the outside of the GUV at 3 sec, resulting in the small vesicles and lipids being dispersed for 1 min (Fig. 2A). They disappeared from eye view at 5 min. On the other hand, Hn-Mc was added to the *E. coli* cells, and the cell surface was observed by SEM. Treatment with the peptide showed small blebs and significant membrane damage to surfaces of the *E. coli* cells, compared to the control, which displayed smooth surfaces without cell debris (Fig. 2B). This is a similar action pattern to the GUV result, indicating that Hn-Mc exhibits antibacterial action by disrupting or damaging the surface membrane.

3.3. Hn-Mc inhibits nitric oxide (NO) and TNF-α release from LPS-stimulated macrophages

AMP can neutralize the endotoxin-induced NO and cytokines release either by binding directly to LPS or by blocking the binding of LPS and LPS-binding protein (LBP) [42,43]. Quantitative evaluations of NO and TNF-α secreted from the murine macrophage, Raw 264.7 cells, were performed to determine the anti-inflammatory activity of the Hn-Mc peptide. As shown in Fig. 3A, LPS-stimulation showed the significant production of NO compared to the control. In contrast, LPS-induced NO secretion in the presence of Hn-Mc was inhibited, indicating the neutralization of LPS-induced NO production by Hn-Mc. The amount of TNF-α, a pro-inflammatory cytokine, released was evaluated using an ELISA kit in the LPS-stimulated cell medium in the presence of the Hn-Mc peptide. Fig. 3A shows that the level of TNF-α secretion was reduced in a concentration-dependent manner. In particular, a treatment of 50 μM reduced cytokine secretion to the control level. In addition, only the peptide treatment without LPS-stimulation reached the same level as the control (Fig. 3A), indicating the non-immunogenicity of Hn-Mc. To examine how Hn-Mc inhibits the LPS-induced pro-inflammatory response, FITC-LPS and Hn-Mc were pre-incubated, and the mixture was then added to the macrophage cells. FITC fluorescence in Raw 264.7 cells with the peptide were weak in a concentration-dependent manner, whereas the cells with only FITC-LPS showed strong fluorescence emission. The result suggests that Hn-Mc prevents the binding of LPS to macrophages. The cationic residues of Hn-Mc could initially bind the phosphate groups of LPS through electrostatic interactions, and Hn-Mc and LPS were further complexed by the hydrophobic residues of Hn-Mc. Therefore, the free LPS that can bind LBP was reduced and macrophages were not stimulated.

3.4. Hn-Mc possesses antibacterial activity in vivo in mouse model infected by drug-resistant bacteria

In general, although AMP has potent antimicrobial activity *in vitro*, it sometimes disappears when applied *in vivo*, due to physiological conditions, such as high salt, Mg^{2+} and Ca^{2+} ions, and

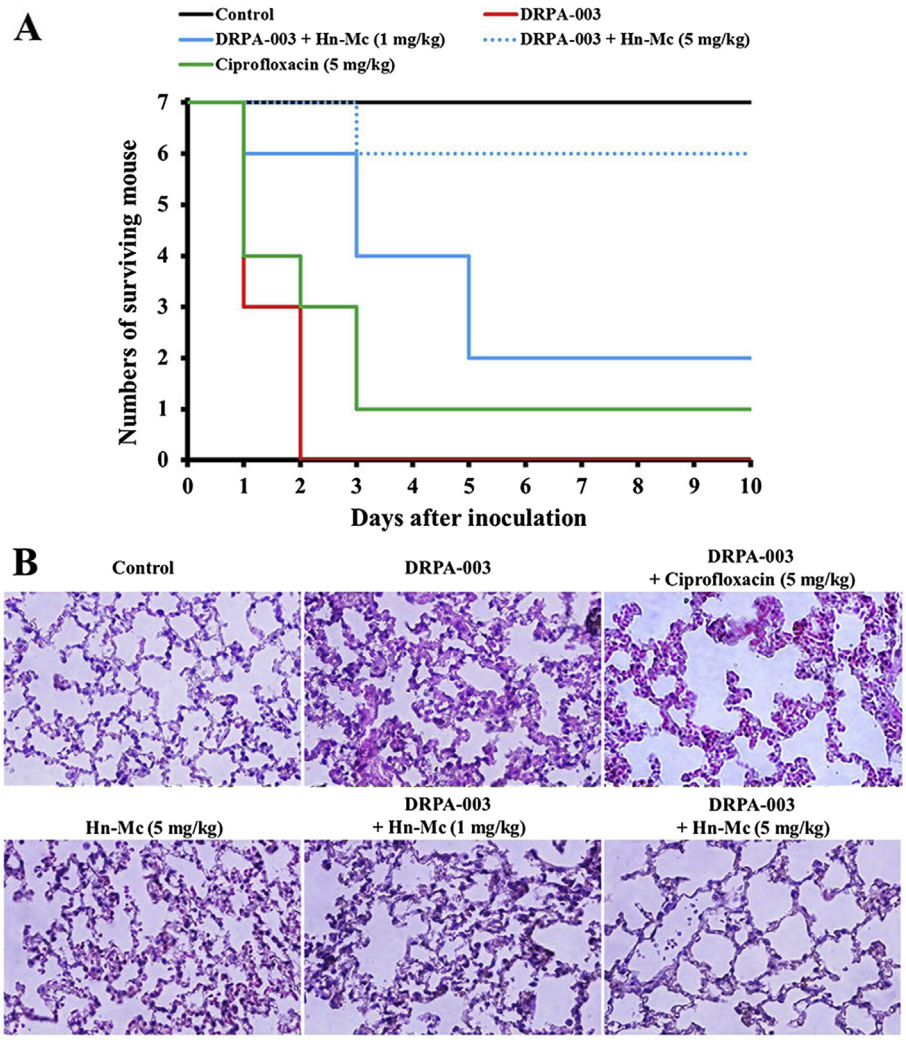

Fig. 4. Evaluation of the antibacterial activity of Hn-Mc against DRPA in a bacterial infection model. To assess the antibacterial effects of Hn-Mc, ICR mice were infected intraperitoneally with DRPA-003. After 1 hr, the samples were intraperitoneally administered at the indicated concentrations and monitored survival for 10 days (A). Histology of DRPA-003 infected mouse lungs stained with H&E (B).

protease. To determine the effects of Hn-Mc on mice infected with drug-resistant bacteria, Hn-Mc was injected at the indicated concentrations after an i.p. injection of DRPA-003, which possesses resistance to ciprofloxacin and gentamicin. The bacteria-infected mice in the absence of Hn-Mc all died within 2 days after inoculation and only one mouse of those given the ciprofloxacin treatment (5 mg/kg) survived. In contrast, 2 (28.6%) and 6 (85.7%) of the bacteria-infected mice given a 1 mg/kg and 5 mg/kg injection of Hn-Mc survived at 10 days. Furthermore, the histopathological examination revealed a marked decrease in the number of swelling cells, vascular leakage and inflammatory infiltrate in the lungs of the DRPA-003 infected mice with Hn-Mc (Fig. 4B). The results highlight the therapeutic potential of Hn-Mc, an *in vivo* animal model. This means that Hn-Mc is resistant to protease and salts, but more study will be needed.

In summary, a novel hybrid peptide, Hn-Mc, with excellent cell selectivity was designed. It possesses the mode of membranolytic action and *in vivo* antimicrobial activity against drug-resistant bacteria. In addition, when the body is infected with drug-resistant bacteria, anti-inflammatory activity is crucial to overcome or prevent death by sepsis, as well as an antimicrobial activity. Overall, the designed chimeric peptide was found to be a model peptide with both antimicrobial and anti-inflammatory activity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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