International Journal of Biological Macromolecules 302 (2025) 140463

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

In vitro and *in vivo* efficacy studies of an engineered endolysin targeting Gram-negative pathogens

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ARTICLE INFO

Keywords:
Engineered endolysin
Gram-negative
Carbapenem-resistant *Acinetobacter baumannii*
Mouse model

ABSTRACT

Endolysins have drawn considerable attention as viable modalities for antibiotic use. The most significant obstacle for Gram-negative targeting endolysins is the presence of the outer membrane barrier. A heterologously expressed endolysin encoded by bacteriophage PBPA90 infecting *Pseudomonas aeruginosa* exhibited intrinsic antibacterial activity against *P. aeruginosa*. The antibacterial efficacy was improved by substituting 15 amino acids and by fusing cecropin A to the N-terminus. The resulting engineered endolysin, LNT103, demonstrated strong antibacterial activity, with minimum inhibitory concentrations as low as 4 μg/ml, against various Gram-negative pathogens in addition to *P. aeruginosa*, including *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella aerogenes*, and *Enterobacter cloacae*. The engineered endolysin rendered both the outer and the inner bacterial membranes permeable. It exhibited a synergistic effect with colistin, and additive effects with carbapenem antibiotics. Bacterial resistance development to LNT103 was none to minimal *in vitro*. Its *in vivo* efficacy was verified in bacteremia models of mice infected with *A. baumannii*. The endolysin led to a resensitization of resistant bacteria to meropenem when used in combination *in vivo*.

1. Introduction

Endolysins are enzymes produced by bacteriophages that degrade the peptidoglycan cell wall *via* a regulated process where large quantities of spontaneously assembled phage particles burst out from inside host bacteria. Phage-encoded proteins, called holins, are directed to the plasma membrane and form a transmembrane multimer complex, which creates a hole [1,2]. Assembled virions then pass through the membrane, only to encounter the second barrier, the peptidoglycan cell wall. The latter cell wall is a thick and rigid structure and enzymes to degrade the barrier are required. This role is performed by phage-encoded endolysins [3–5]. The cell wall-degrading activities of endolysins render these enzymes antibacterial [6]. Endolysins may possess muralytic activities, with examples including amidase, endopeptidase, lytic transglycosylase, or glucosaminidase. When recombinant endolysins are applied from outside the cell, the peptidoglycan cell wall of a bacterium can be instantly degraded upon contact, especially in the case of Gram-positive bacteria. Several endolysins targeting Gram-positive *Staphylococcus aureus* are currently under clinical evaluation [7–9].

In Gram-negative bacteria, endolysins from outside a cell make first contact with the outer membrane, not the cell wall. Accordingly, they must penetrate the former membrane to find and then degrade the peptidoglycan cell wall. Many endolysins targeting Gram-negative bacteria that have been characterized to date lack antibacterial

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https://doi.org/10.1016/j.ijbiomac.2025.140463
Received 14 November 2024; Received in revised form 9 January 2025; Accepted 27 January 2025
Available online 28 January 2025

efficacy [10]. Five different globular endolysins from bacteriophages infecting *Klebsiella* were reported to have muralytic activities against Gram-negative bacteria only in the presence of the outer membrane permeabilizer, EDTA [11]. Another endolysin from *Klebsiella* phage KP27 was also shown to possess peptidoglycan-degrading activity on membrane-permeabilized bacteria [12]. Nonetheless, some exceptional cases have been reported. LysPA26 demonstrated endolysin activity against *Pseudomonas aeruginosa* without pretreatment with an outer membrane permeabilizer [13]. Another endolysin, Ply6A3, was shown to have efficacy against *Acinetobacter baumannii* both *in vitro* and *in vivo* [14]. A 2 to 3.7 log-reduction of various Gram-negative bacterial loads was reported with endolysin Ts2631 from *Thermus scotductus* phage vB_tsc2631 [15]. Also, endolysin LysSS was effective against various Gram-negative pathogens both *in vitro* and *in vivo* [16]. Yet another endolysin, LysAB54, was seen to possess bactericidal activity against various Gram-negative pathogens resuspended in buffer, though activity was not seen in a complex medium or in human serum [17]. The difficulty in finding effective endolysins against Gram-negative bacteria has led to the development of engineered Artilysins, where Artilysins combining a polycationic nonapeptide for penetration of the outer membrane and a modular endolysin were able to kill Gram-negative multidrug-resistant (MDR) strains *in vitro* with a 4- to 5-log reduction within 30 min [18,19].

Other engineered endolysins have also been reported on. Cecropin A is the most used antimicrobial peptide (AMP) for enhancing the antimicrobial efficacy of engineered endolysins. Cecropin A-fused endolysin LysMK34, LNT113, 1D10, and PBST08 were seen to be effective against *A. baumannii* [20–24]. The N-terminal 8 amino-acids derivative (KWKLFKKI) from cecropin A was also used for the same purposes [25–27]. Furthermore, a previous study, where peptides derived from the endolysin LysAB2 encoded by the *A. baumannii* phage fAB2 displayed a potent antibacterial activity against *A. baumannii* (MIC, 4 μM), demonstrating that part of an endolysin could be selectively used as an antimicrobial peptide [28]. Similarly, the fusing of a sensitizing peptide, KL-N9P, improved the efficacy of a native endolysin against various Gram-negative pathogens [29]. Strategies for overcoming the outer membrane barrier of Gram-negative pathogens have been previously well reviewed [30,31].

With emphasis on the development of an endolysin as a novel antibacterial drug, we report on the *in vitro* and *in vivo* characterization of an engineered endolysin against various Gram-negative pathogens. In particular, comparisons with currently utilized antibiotics, and the limitations of mouse infection models used for the evaluation of antibacterial efficacy, have been explored in depth.

2. Materials & methods

2.1. Bacteriophages and endolsyins

Bacteriophage PBPA90 infecting *P. aeruginosa* PAO1 was obtained from the Bacteriophage Bank of Korea (www.phagebank.or.kr). The phage was isolated from a wastewater facility in Gwacheon in the Republic of Korea ('Korea') and was selected for further development based on the endolysin's initial antibacterial efficacy.

2.2. Bacterial strains and culture conditions

ATCC strains were procured from the American Type Culture Collection (ATCC). KCTC strains were obtained from the Korean Collection for Type Cultures (KCTC, Korea, http://knrrb.ccarm-bio.or.kr/index.jsp?rrb=ccarm). CCARM strains were sourced from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Korea, http://knrrb.ccarm-bio.or.kr/index.jsp?rrb=ccarm). F strains, which were gifts kindly provided by Professor Kwan Soo Ko (Sungkyunkwan University School of Medicine, Korea), were clinically isolated. The colistin-resistant FORC81 strain was also generously provided by Professor Sangryeol Ryu (Seoul National University, Korea). All strains were grown in LB (Luria-Bertani) broth, CAA medium (5 g/L casamino acids, 5.2 mM K_2HPO_4, and 1 mM $MgSO_4$), or MHB (2 g/L beef infusion, 1.5 g/L starch, 17.5 g/L casein hydrolysate) at 37 °C.

2.3. Molecular cloning

The endolysin (GenBank accession no. MW815133) from bacteriophage PBPA90 was named LNT101. For amino acid substitution, 14 closely related sequences observed through a BLAST search are illustrated in Supplementary Table 1. A derivative, LNT102, was generated by substituting 15 amino acids by gene synthesis (Bioneer, Korea) (Table 1). Another derivative, LNT103, was constructed by fusing the antimicrobial peptide cecropin A (full length, GenBank accession no. 0708214A) to the 5′ end of LNT102 with a GS linker. The coding sequence of LNT103 was amplified by PCR. An LNT103 gene fragment was inserted between the *Nhe*I and *Xho*I restriction sites of pET21a + (Merck, Germany), with the resulting recombinant protein having a C-terminal His-tag.

2.4. Recombinant protein expression and purification

Endolysin expression vectors were introduced into *E. coli* BL21 Star (DE3) (Invitrogen, USA) using heat shock transformation. The cells were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG; Duchefa, the Netherlands) at a concentration of 0.5 mM at 25 °C for 5 h. The bacterial cells were harvested by centrifugation and the pellets resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 20 mM Imidazole). The resuspended cells were disrupted by homogenizer. After centrifugation at 8,000 xg at 4 °C for 20 min, the supernatant was recovered and filtered through 0.2 μm pores. The filtered supernatant was purified using FPLC (AKTA pure, Cytiva, UK).

A Ni-His tag affinity chromatography (Capto Chelating, Cytiva) was performed as the first process and eluates were collected using 500 mM imidazole. The eluates were diluted with an equilibration buffer (20 mM sodium phosphate, pH 7.5) and then loaded on a cation exchanger (Capto S, Cytiva). Elution from the cation exchange column was performed with a linear gradient of NaCl from 0 to 1 M in 20 mM sodium phosphate, pH 7.5. Buffer exchange of the eluate was performed by ultrafiltration and diafiltration in an equilibration buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and the mixture was loaded onto an anion exchanger (Capto Q, Cytiva) to remove endotoxins. The eluate was mixed with 20 mM Tris-HCl, 2 M ammonium sulfate, pH 7.5. Then, the mixture was subjected to hydrophobic interaction chromatography (Butyl HP, Cytiva). Elution was performed with a linear gradient of ammonium sulfate from 1 to 0 M in 20 mM Tris-HCl, pH 7.5. Finally, buffer exchange of the eluate was performed by ultrafiltration and

Table 1
Substituted amino acids and their position from the endolysin LysPBPA90 (LNT101) to LNT102.

Amino acid position	LNT101	LNT102
22	R	N
27	A	N
28	V	L
45	F	V
48	S	G
49	K	A
50	A	G
60	A	K
63	A	Y
64	E	A
65	L	I
103	T	V
125	S	K
147	H	Y
176	L	I

diafiltration in a storage buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.0). As a control, full length cecropin A (KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2) was obtained *via* the peptide synthesis service (AbClon, Korea).

2.5. Gel-clot assay for endotoxin

Quantitative analysis of endotoxin in purified LNT103 was performed using Endosafe® Gel-clot LAL Vial at a sensitivity of 0.03 EU/ml (R12003, Charles River, USA). LNT103 was prepared by 4-fold dilution with endotoxin-free water. Control Standard Endotoxin (CSE) at 0.0625 EU/ml served as a positive control and endotoxin free water was used as a negative control. Samples in dedicated tubes were mixed with Limulus amoebocyte lysate (LAL) reagent and incubated at 37 °C for 1 h. Following the reaction, the tubes were immediately inverted to confirm whether a gel had formed. All tests were performed in duplicate. The final endotoxin limit of LNT103 was calculated using the following equation: Endotoxin limit (EU/mg) = Sensitivity of LAL reagents (0.03 EU/mg) x dilution factor / concentration of LNT103. The endotoxin content of purified LNT103 (section 2.4) was found to be <0.005 EU/mg.

2.6. Antibacterial activity assay (Viable cell counting)

The antibacterial activity of the purified protein was assessed for various Gram-negative bacteria. Briefly, bacteria were grown to an exponential phase (OD_{600} = 0.5), washed with reaction buffer (20 mM HEPES-NaOH, pH 7.4), and diluted in the buffer to approximately 10^6 cells/ml. Then, 100 μl of the bacterial suspension was mixed with 100 μl of the purified endolysin and/or colistin in reaction buffer and the mixture incubated at 37 °C for 2 h. Finally, the mixture was diluted with PBS and plated on LB agar. After overnight incubation at 37 °C, colonies were counted.

For salt stability, the same reaction was carried out in 20 mM HEPES-NaOH, with 0, 50, 150, 300, or 500 mM NaCl (pH 7.4). For thermal stability, LNT103 was incubated at different temperatures (4, 25, 37, 50, 60 or 70 °C) for 1 h followed by antibacterial activity assay against *A. baumannii* ATCC 19606 in reaction buffer (20 mM HEPES-NaOH, 150 mM NaCl (pH 7.4)). The antibacterial activity for all results is represented as a percentage of relative activity compared to the control (4 °C). To test the effect of pH, the reaction was performed in 20 mM sodium phosphate, 150 mM NaCl, adjusted to different pHs (6, 6.5, 7, 7.5, or 8). The cells suspended in the buffer for each pH were mixed with LNT103 and incubated at 37 °C for 1 h. For the control, cells were suspended in HEPES-NaOH buffer, 150 mM NaCl, pH 7.4 mixed with LNT103 and incubated at 37 °C for 1 h.

2.7. Permeability test of bacterial membranes

Escherichia coli ML-35p harboring pBR322 produces β-lactamase in the periplasmic space and β-galactosidase in the cytosol [32,33]. The disruption of the outer and inner membranes was measured by colorimetric changes of nitrocefin (Abcam, Cambridge, UK) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG; KisanBio, Seoul, Korea), respectively. *E. coli* ML-35p cells (10^8 cells/ml) at the mid-logarithmic growth phase were treated with different concentrations of endolysins (0–0.6 μM) in 5 mM HEPES buffer (pH 7.2). Polymyxin B (Sigma-Aldrich, St. Louis, MO, USA) was used at 1 μM as a control to disrupt the membranes. Nitrocefin at 50 μg/ml or ONPG at 1.5 mM were added to the bacterial suspension and the chromogenic changes were determined at 486 nm or 420 nm, respectively, using a Synergy HTX microplate reader (BioTek, Paramus, NJ, USA).

2.8. Measuring the minimum inhibitory concentration (MIC)

Except for the growth media, the MIC of LNT103 and the various antibiotics were determined by the broth microdilution method in 96-well, round-bottomed microtiter plates in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. The exponentially grown cells were diluted in CAA media (10^6 CFU/ml) and then incubated with recombinant LNT103 (1 to 64 μg/ml) or antibiotics (0.5 to 32 μg/ml) at 37 °C for 20 h. The MIC was defined as the lowest antibacterial concentration that inhibited bacterial growth.

2.9. Checkerboard assay

A checkerboard assay was performed by methods previously described [34,35]. In short, in a 96-well, round-bottomed microtiter plate, the two-fold serial dilution of LNT103 was aliquoted along the wells at the y-axis. In another microtiter plate, a two-fold serial dilution of colistin was aliquoted along the wells at the x-axis. The two diluents were combined in a single plate, and bacteria were added to each well at a concentration of 10^6 CFU/ml in MHB media. After incubation at 37 °C for 20 h, the MIC values were recorded. The fractional inhibitory concentrations (FIC) for LNT103 or antibiotics were calculated as the MIC value in the presence of two drugs divided by the MIC value in the presence of each drug. If the sum of the two FICs (FIC index) was ≤0.5, the two drugs were considered to have a synergistic effect.

2.10. Development of bacterial resistance

To test the increase of MIC due to resistance development, bacteria were continuously exposed to colistin or endolysin by means previously described [36]. Briefly, the initial MICs of colistin and LNT103 were determined against various strains of *A. baumannii*. Then, bacteria were cultured in MHB media containing half the concentration of MICs at 37 °C. After 24 h, the MICs were determined again. Then, 1:100 diluted bacterial cells were cultured in a fresh medium containing LNT103 or colistin. This process was repeated for 15 passages. The MICs were measured and recorded at each passage.

2.11. Biofilm removal assay

In a 96-well, round-bottomed plate, *A. baumannii* ATCC 19606 cells were inoculated at 10^6 cells/well in LB media and grown at 37 °C for 24 h to allow for biofilm formation. The culture was discarded and each well washed twice with PBS, followed by treatment with LNT103 or colistin in a reaction buffer (20 mM Tris-HCl, pH 7.5). After 1.5 h, each well was washed with PBS and stained with 0.5 % crystal violet for 30 min. The dye was dissolved by adding 150 μl of 100 % ethanol and the OD was measured at 590 nm using a microplate reader.

2.12. Confocal laser scanning microscopy imaging of biofilms

A. baumannii ATCC 19606 cells (~10^8 CFU) were placed on slides and cultured at 37 °C for 24 h. The following day, the culture supernatant was discarded, and the slides were dried for 10 min. The slides were then washed with PBS. After drying for 10 min, each test substance was added and incubated at 37 °C for 2 h. The staining reagent (Live/Dead Bacterial viability kit L7012, ThermoFisher, USA) was added to each slide and incubated in the dark for 30 min. Viable cells were stained green (SYTO 9) and damaged cells were stained red (propidium iodide). Next, the staining reagents were removed, and the slides were washed with PBS. The dried slides were then covered with coverslips and observed under a confocal laser scanning microscope (Leica, Germany). *Z*-stack images were created using the Leica Application Suite X software package (Leica) and images were merged and reconstructed in 3D.

2.13. Hemolysis assay

In vitro hemolytic activity testing of the recombinant endolysins was performed in sheep red blood cells (RBCs). First, 3 ml of RBCs (MB Cell,

Korea) was washed four times with phosphate-buffered saline (PBS) at pH 7.2 and resuspended in 10 ml PBS. Then, 50 μl of RBC solution was added to 50 μl of LNT103 (2–128 μg/ml), PBS (negative control), or 0.1 % Triton X-100 (positive control) and the mixtures were incubated at 37 °C for 1 h. The mixtures were then subjected to centrifugation (1000 xg, 5 min) and the supernatants were transferred to 96-well plates. Absorbance was measured at 570 nm. The percentage of hemolysis was calculated using the following equation: % Hemolysis = {[Abs (sample) – Abs (negative control)] / [Abs (positive control) – Abs (negative control)]} x 100.

2.14. In vitro cytotoxicity assay

To test the cytotoxicity of recombinant LNT103, a human hepatocellular carcinoma cell line (Huh-7) was used. First, 1×10^9 cells were seeded in 96-well plates. Then, LNT103 was added to a final concentration of 0.25 or 0.5 mg/ml and the mixtures were incubated for 24 or 48 h. PBS and 1 % Triton X-100 were used as negative and positive controls, respectively. After incubation, 10 μl of tetrazolium salt solution, using a D-Plus™ CCK Cell Viability Assay Kit (Dongin Bio, Korea), was added to each well. The production of formazan was measured at 450 nm.

2.15. Statistical analysis

For *in vitro* studies, all experiments were carried out in triplicate and the results were given as the mean ± standard deviation (SD). Statistical analysis was performed in Prism version 9 (GraphPad, Dotmatics). NPN assay and CFU reduction assay were analyzed using one-way ANOVA with Tukey's multiple comparisons test.

2.16. Mouse infection models

All animal experiments were approved by the Animal Ethics Committee of Kangwon National University, Korea (KW-231019-4). 6-week-old BALB/c female mice were used. Mice were pre-treated (intraperitoneal) with cyclophosphamide (in 100 μl PBS) at a concentration of 3 mg/mouse at four days and one day before the bacterial inoculation to induce neutropenia [37–39]. Either 2×10^8 CFU (*A. baumannii* ATCC19606) or 1×10^7 CFU (*A. baumannii* CCARM12233) in 100 μl PBS were intraperitoneally injected and the endolysin LNT103 in 100 μl PBS was treated with IV bolus at 1 h post infection. The amount of each *A. baumannii* strain used for inoculation was empirically determined as the minimal number of bacteria which could lead to animal death (Supplementary Fig. 1). Meropenem (Sigma, USA) at the indicated concentration in 30 μl PBS was treated SC at 1 h post infection as required. For enumerating the blood bacterial burden, peripheral blood samples (100 μl) were collected from the retro-orbital venous sinus at the indicated time points post-infection. The blood was diluted four-fold in PBS, plated on LB agar plates containing trimethoprim (2.5 μg/**ml**), and incubated at 37 °C for 18 h. The bacterial burden was quantified by calculating CFU/**ml** from the plated blood samples. For quantifying serum cytokine levels, serum was obtained by subjecting 100 μl of blood samples collected at 15 h post infection from *A. baumannii*-infected mice to centrifugation for 30 min at 3000 ×*g* at 4 °C. The levels of IL-6 and TNF-alpha in the serum were quantified using the Mouse IL-6 Uncoated ELISA Kit (Invitrogen #88–7064-88) and the Mouse TNF-α Uncoated ELISA Kit (Invitrogen # 88–7324-88), respectively. Prior to collection, the mice were anesthetized with isoflurane to minimize stress and discomfort. The animals were sacrificed using CO_2 inhalation.

2.17. Anti-drug antibody tests

Anti-drug antibody testing was performed at BioToxTech, Korea. All procedures in this study were fully in compliance with the Animal Protection Act of Republic of Korea (2011, as amended) and the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011). Nine 6-month-old male and nine 6-month-old female beagle dogs were obtained from Jiangsu Zhaoshengyuan Biotechnology Co., Ltd., the People's Republic of China. The dogs were divided into three groups and a daily dose of 0, 3, or 10 mg/kg of LNT103 was intravenously administered, respectively. Blood collection and serum separation for ADA analysis were performed on all of the animals at days 0 and 15. Blood samples of approximately 2.5 **ml** were collected from the jugular vein using a 3 ml disposable syringe and placed in SST bottles and centrifuged at 3000 rpm for 10 min at 4 °C to obtain serum. An analysis of ADA levels was performed by Keyfron Bio Co., Ltd. (Korea).

3. Results

3.1. Endolysin LysPA90 and its engineering

LysPA90 (LNT101) is based on an ORF annotated as an endolysin (GenBank accession no. MW815133) from the genome of bacteriophage PBPA90 (Fig. 1A). It consists of a peptidoglycan binding domain at the N-terminus (amino acids 10–65) and a muramidase (lytic transglycosylase) domain (amino acid 95–174) in the middle of the ORF. It harbors an amphipathic helix at its C-terminus (amino acids 298–311, LAEIYKLF (hydrophobic)/ EDKVSK (hydrophilic), Fig. 1B), which is a common feature of endolysins exhibiting intrinsic antibacterial activity against Gram-negative pathogens [40,41].

We subsequently attempted to improve its activity. First, site-directed mutagenesis was performed based on amino acid sequences found in 14 closely related entities from a BLAST search. Six were annotated as endolysins (ANM44938.1, NP_803710.1, YP_009619739.1, YP_006546280.1, AUG88272.1, and YP_009609055.1) and eight were annotated as peptidoglycan binding proteins (YP_009609112.1, QBJ02724.1, QEM41943.1, YP_007349105.1, ASZ78903.1, AYP28388.1, QIQ61961.1, and AVO22912.1) (Supplementary Table 1). Based on an assumption that a frequently found amino acid at a specific position reflects an evolutionary advantage, we performed site-directed mutagenesis at 15 such positions (Table 1). For the substitutions, the rationale was to select the most commonly found amino acids at the indicated positions for CBD from the eight peptidoglycan binding proteins, and the mostly found amino acids at the indicated positions for EAD from the six endolysins (Supplementary Fig. 2). The resulting mutant endolysin demonstrated much-improved activities (more than a log reduction in bacterial counts) against *A. baumannii* and *K. pneumoniae* (Fig. 1D) and was named LNT102. A further improvement of LNT102's antibacterial activity was obtained by fusing an antimicrobial peptide called cecropin A to its N-terminus (Fig. 1A and D), resulting in LNT103. The three endolysins purified to near homogeneity are illustrated in Fig. 1C. Varying degrees of antibacterial efficacies were observed, ranging from <0.5 log to 6 log reductions of viable cells. The antibacterial efficacy of endolysin LNT103 against a wider range of bacterial strains, including antibiotic resistant ones, are provided in Supplementary Fig. 3A and B. As expected, the antibacterial-resistant nature of the target strain did not affect the endolysin's antibacterial efficacy. Cecropin A itself exhibits antibacterial efficacy, but LNT103 demonstrated a further substantial increase in efficacy *in vitro*.

Fig. 2 shows the predicted structures of LNT101 and LNT102. The 15 amino-acid substitutions led to a structural change in the N-terminal cell wall binding domain (Fig. 2A). The orientation of 3 α-helices at the N-terminus was altered and it also led to a change in the overall orientation of N- and C-termini. The substitution resulted in an increase in the positive charge in the N-terminus (Fig. 2B), possibly leading to a stronger interaction with the bacterial membrane.

3.2. In vitro antibacterial efficacy of LNT103

We examined the contribution of cecropin A to LNT102 for the antibacterial efficacy of LNT103, namely the fusion of cecropin A to

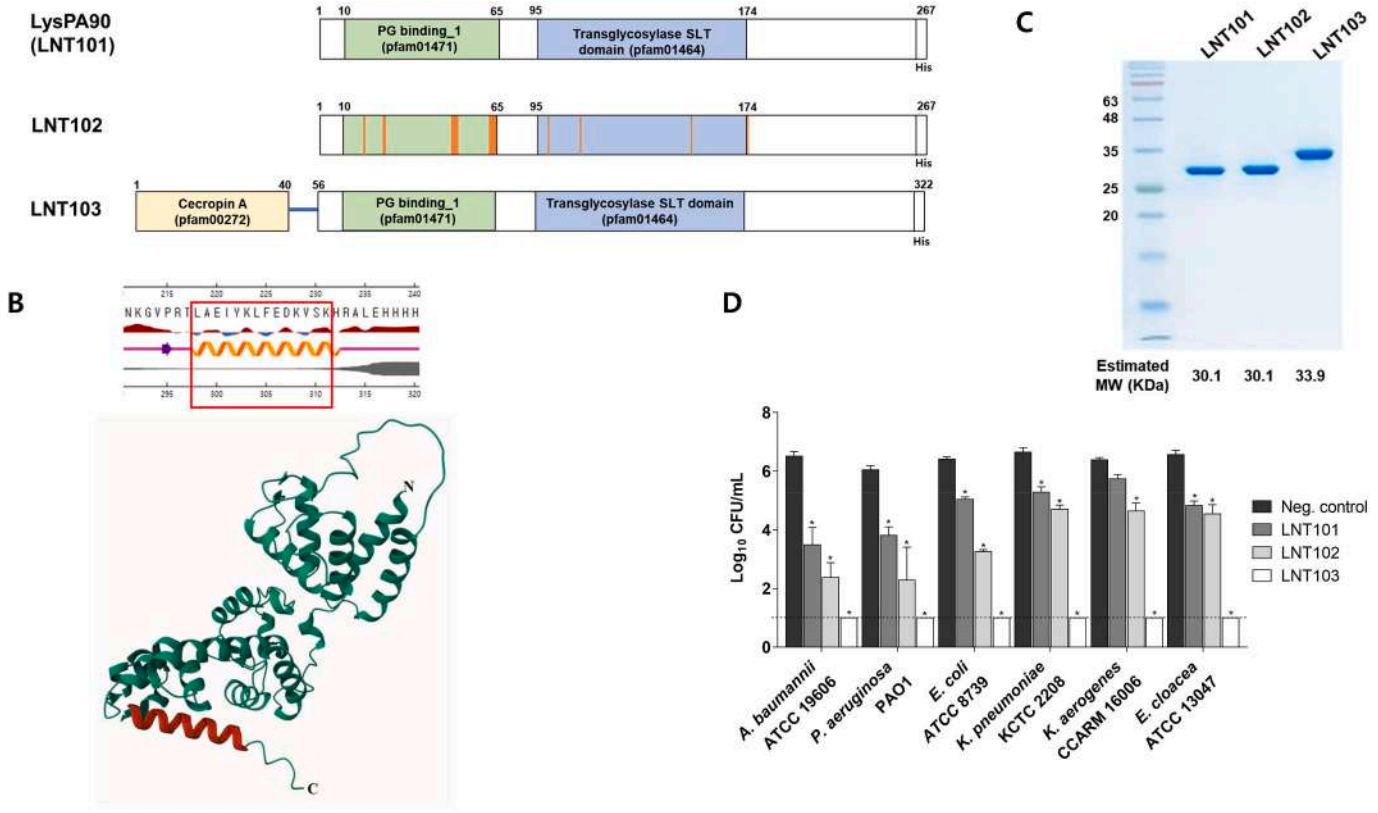

Fig. 1. Improvements of antibacterial activity of the endolysin PA90 (LNT101)
(A) A peptidoglycan binding domain (PG domain) and a muramidase domain are illustrated. The numbers above each box indicate the amino acid residue of the enzyme. Those amino acids subjected to replacement in LNT102 are shown with vertical bars (orange) (and provided in Table 1). LNT103 has cecropin A fused to the N-terminus of LNT102. (B) A predicted amphipathic helix shown between amino acids 299 and 302 in LNT101 (LysPA90) using NetSurf 3.0 (https://services.healthtech.dtu.dk/services/NetSurfP-3.0). 3D structure is predicted using ColabFold v1.5.5 (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb#scrollTo=G4yBrceuFbf3) and drawn using 3D viewer Mol* (Protein Data Base, https://www.rcsb.org/docs/3d-viewers/mol*/getting-started). The amphipathic helix at the C terminus is indicated in red. (C) SDS-PAGE analysis of purified endolysins LNT101, LNT102, and LNT103. The estimated molecular weight of 30.1 KDa (LNT01 and LNT102) and 33.9 KDa (LNT103) are shown at the bottom. The molecular weight marker is seen in the left lane. (D) Antibacterial activities of LNT101, 102, or 103 (1 μM for 1 h) against various Gram-negative bacteria (** $p < 0.005$, * $p < 0.05$). CCARM strains were antibiotic-resistant and obtained from the Culture Collection of Antimicrobial Resistant Microbes. For the control, no endolysin was added to the mixture. Three independent experiments were performed. Statistical analysis was conducted against the control in each bacterial group.

LNT102 (Fig. 3A). Cecropin A alone reduced the bacterial count by 3.5 logs while fusion of EGFP to cecropin A completely abolished antibacterial activity. LNT102 alone reduced the bacterial count by 1 log, and addition of cecropin A further reduced the bacterial count by 4 more logs. LNT103 reduced the bacterial count 2 logs more than cecropin A alone and 4 logs more than LNT102. Accordingly, the fusion of the antimicrobial peptide and the endolysin led to a synergistic increase in antibacterial efficacy. Since colistin-resistant strains are increasingly reported on [42], we tested whether LNT103 was also effective against one of these strains, *E. coli* FORC81 (Fig. 3B). Colistin did not reduce the bacterial count at concentrations of 0.5 and 2 μM, while LNT103 reduced the bacterial count by 4 logs at 0.5 μM and to a below countable limit at 2 μM in 120 min. We also tested whether LNT103 was effective for removing bacteria in biofilms (Fig. 3C). It was seen to remove bacteria in a dose-dependent manner, leading to a 65 % removal at 3 μM, while colistin did not result in any removal at 3.6 μM. The same comparison was shown in a confocal microscopic analysis of biofilms treated with either colistin or the endolysin (Fig. 3D). The biofilm remained intact with treatment of colistin at 4 μM, while the endolysin removed the biofilm in a dose-dependent manner. The MICs of LNT103 against 100 different strains of *A. baumannii* are listed in Supplementary Table 2, where MIC_{50} was 4 μg/ml. MIC_{50} for 62 strains of *K. pneumoniae* was 4 μg/ml, MIC_{50} for 100 strains of *E. coli* was 8 μg/ml, and MIC_{50} for 84 strains of *P. aeruginosa* was 8 μg/ml (data not shown).

3.3. Cecropin A fusion facilitated the disruption of the double-membraned envelope

The permeability of the outer membrane increased more when treated with LNT103 than with LNT102 (Fig. 4A). But most notably, the time needed for reaching the highest value was much shorter with LNT103 (6 min) than with LNT102 (16 min). Thus, the permeability of the inner membrane increased up to 2 folds more in 30 min with LNT103 than with LNT102 (Fig. 4B) and the action was also quicker with LNT103. The increases were in a dose-dependent manner. Polymyxin B was less effective than the endolysins LNT102 or LNT103, being far less effective in permeabilizing the inner membrane than the outer membrane. Permeabilization of the outer membrane may enable entrance of the endolysin leading to cell wall destruction, while permeabilization of the inner membrane may further damage the bacteria by disrupting membranepotential leading to death.

3.4. Biochemical nature of the antibacterial activity

The endolysin LNT103 demonstrated a time-dependent activity against *A. baumannii* (Fig. 5A). For *A. baumannii*, the bacterial count decreased 3 logs in one minute and to a below detectable level at 60 min post treatment with the endolysin at 0.2 μM. When comparing with colistin, the endolysin reduced the number of target bacteria remarkably

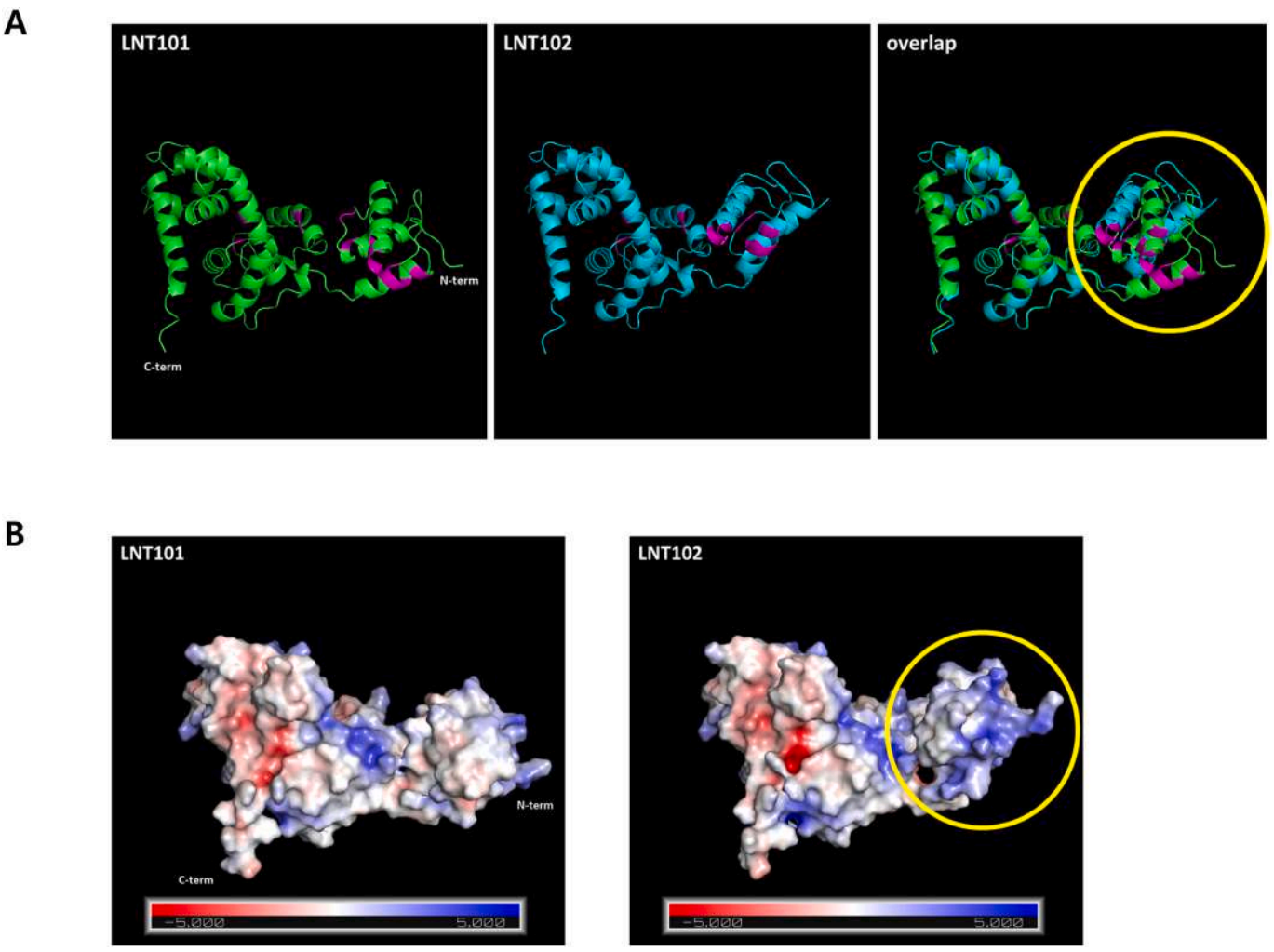

Fig. 2. Structural predictions of LNT101 and LNT102
(A) Alphafold 2-predicted structures of LNT101 (green) and LNT102 (blue) are indicated. Substituted amino acids are shown in violet. The altered orientation of 3 α-helices is marked by a yellow circle. (B) Comparison of charge distribution between LNT101 and LNT102. Positive charge is shown in blue and negative charge in red. An increased positive charge in the N-terminus is marked by a yellow circle.

faster (Supplementary Fig. 4). Next, we observed changes in antibacterial efficacy under various salt, pH, and temperature conditions. The antibacterial activity of the endolysin started to decrease in the presence of NaCl at 150 mM and was completely lost in the presence of NaCl at 500 mM (Fig. 5B). Since the physiological NaCl concentration of human serum is 150 mM, we can expect the loss of activity to a certain extent *in vivo*. The enzymatic nature of the protein would lead to decreases or increases of activity in the presence of various levels of salts. Nevertheless, *in vivo* efficacy was confirmed in the mice infection models (see section 3.8). The antibacterial activity of the endolysin remained constant at physiological pHs (6 to 8, Fig. 5C). The enzyme remained stable when incubated at temperatures of up to 50 °C for one hour, but lost 60 % of its activity at 60 °C (Fig. 5D).

3.5. Development of bacterial resistance against LNT103

Although use of colistin is very restricted due to its nephrotoxicity and neurotoxicity, it remains a last resort Gram-negative antibiotic. However, the emergence of colistin-resistant pathogens has already been reported [43–45]. To compare the development of resistance against colistin or LNT103, 15 serial passages of various strains of *A. baumannii*, including both antibiotic-resistant strains and clinically isolated strains, were performed in the presence of a sublethal dose of colistin or LNT103. For *A. baumannii* ATCC19606, a colistin resistance began to develop in the 9th passage, increasing to >256 times the initial MIC in the 12th passage, and then leveling out until the final 15th passage (Fig. 6A). For *A. baumannii* CCARM12171, a colistin resistance began to develop in the 10th passage, then increasing to >512 times the initial MIC by the 13th passage (Fig. 6B). For CCARM12035, a colistin resistance began to develop in the 4th passage, and reached 64 times the initial MIC by the 8th passage. (Fig. 6C). For the clinical strain TA5, a colistin resistance began to develop in the 3rd passage, and reached >512 times the initial MIC by the 6th passage. (Fig. 6D). In all strains, resistance to the endolysin LNT103 was not detected after 15 passages.

3.6. Cytotoxicity and hemolytic activity of LNT103

No decrease in the viability of Huh-7 cells treated with LNT103 at the concentrations of 250 or 500 μg/ml was observed in 48 h, while >99 % of cell death was seen when treated with Triton X-100 at a concentration of 1 % (*w/v*) (Fig. 7A). <1 % hemolysis was observed when treated with LNT103 at concentrations of 2–128 μg/ml, while treatment with Triton X-100 at a concentration of 0.1 % (w/v) resulted in 100 % lysis of RBCs (Fig. 7B). Thus, no significant cytotoxicity or hemolytic activity of LNT103 was shown *in vitro*.

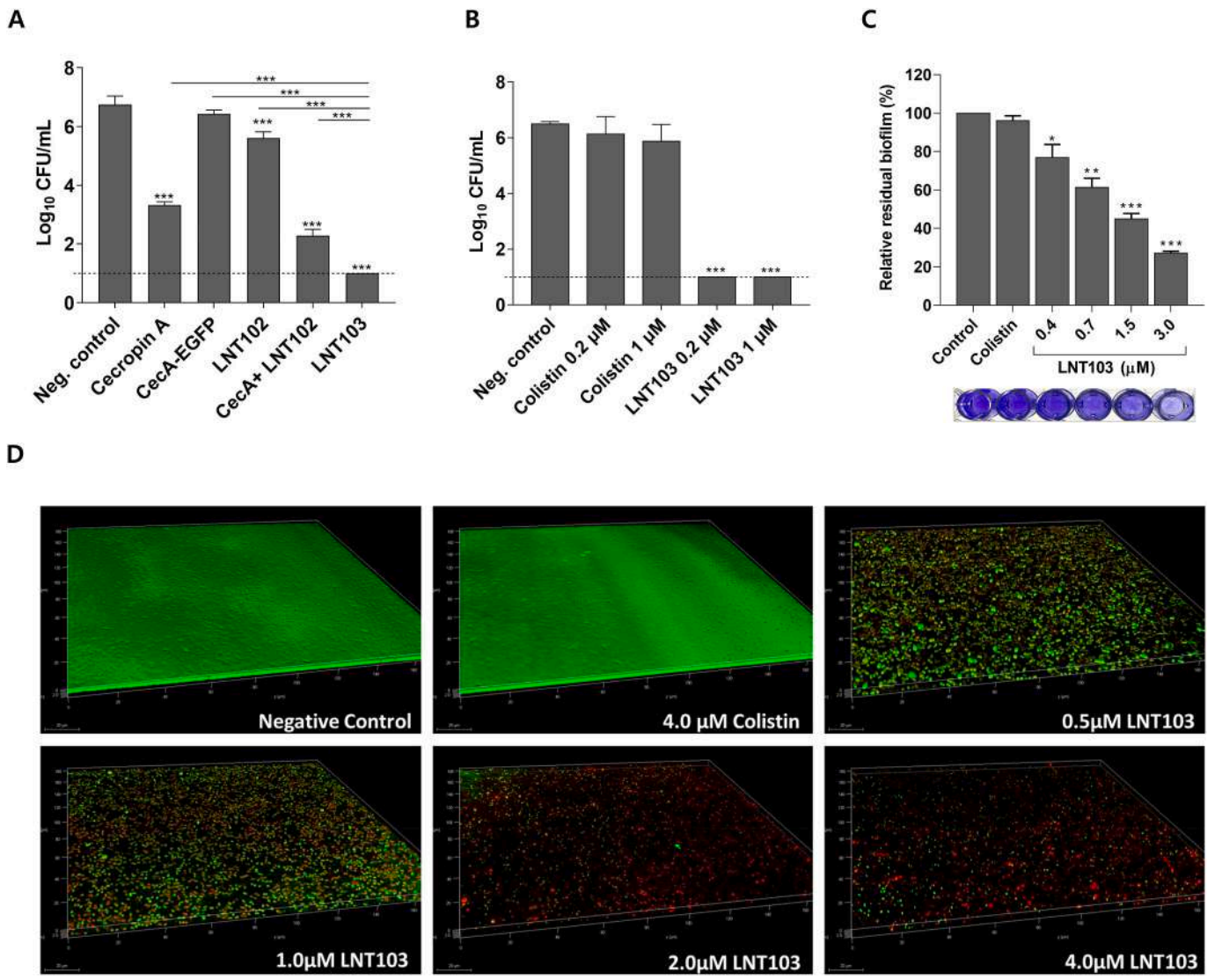

Fig. 3. Antibacterial efficacy of LNT103 *in vitro*
(A) Antimicrobial efficacy of cecropin A, cecropin A-fused to EGFP, or LNT103 against *A. baumannii*. Each reagent was added to the mixture at a final concentration of 0.5 μM. Three independent experiments were performed. The results of *t*-tests against each control are shown above each bar and that of one-way ANOVA is displayed at the top (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Antimicrobial efficacy of LNT103 against a colistin resistant strain of *E. coli*. (C) Biofilm of *A. baumannii* ATCC 19606 treated with LNT103 at four different concentrations (0.4–3.0 μM) or colistin (3.6 μM) for 4 h and visualized by staining with crystal violet. (D) Control indicates the sample treated with buffer only. Dotted lines; limit of bacterial count. Every experiment was performed in triplicate. Student's t-test was performed against each control and *p* values can be seen above each bar (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.7. Combination effect of LNT103 and antibiotics

To assess possible synergistic or additive effects between LNT103 and antibiotics, checkerboard assays were performed (Table 2). Five antibiotics currently used for MDR *A. baumannii* were selected for testing; colistin, meropenem, imipenem, ceftazidime and tigecycline [46,47]. Colistin displayed a strong synergy with the endolysin, suggesting membrane permeability would be a major contributing factor. Two carbapenem antibiotics, meropenem and imipenem, and a cephalosporin antibiotic, ceftazidime, demonstrated additive effects, suggesting cell wall synthesis inhibition by the antibiotics was positively affected by the membrane permeabilization and/or cell wall degradation in the presence of the endolysin. Tigecycline, a translational inhibitor, had an indifferent effect when combined with the endolysin.

3.8. In vivo efficacy of LNT103 against A. baumannii in mice models

Laboratory mice with induced neutropenia were intraperitoneally infected with *A. baumannii* ATCC19606 and IV-treated with LNT103 at two different doses (Fig. 8A). At 10 mg/kg, the endolysin treatment increased survival to 55 % while all mice in the control group died on the first day. The blood concentration of *A. baumannii* in the same mice model was examined (Fig. 8B). Blood bacterial titer was 10^6 CFU/ml at 3 h post infection and increased to 10^8 CFU/ml at 9 h. When compared to the bacterial burden at 10^3 to 10^4 CFU/ml in usual human blood stream infections (BSIs) [48], the number of bacteria in this mice model was 100 to 1000 times higher. For examining the combination effect of antibiotics and the endolysin, laboratory mice were intraperitoneally infected with *A. baumannii* CCARM12233, a carbapenem-resistant strain, and treated with either meropenem alone or in combination with endolysin LNT103 (Fig. 8C). All mice treated with low dose meropenem (16 mg/kg) died and even 90 % of those treated with a high dose of the antibiotic (64 mg/kg) died within three days. By contrast, mice treated with a small amount of the endolysin (0.625 mg/kg) in addition to meropenem had increased survival rates of 45 % (with low dose meropenem) and 60 % (with high dose meropenem). Thus, the

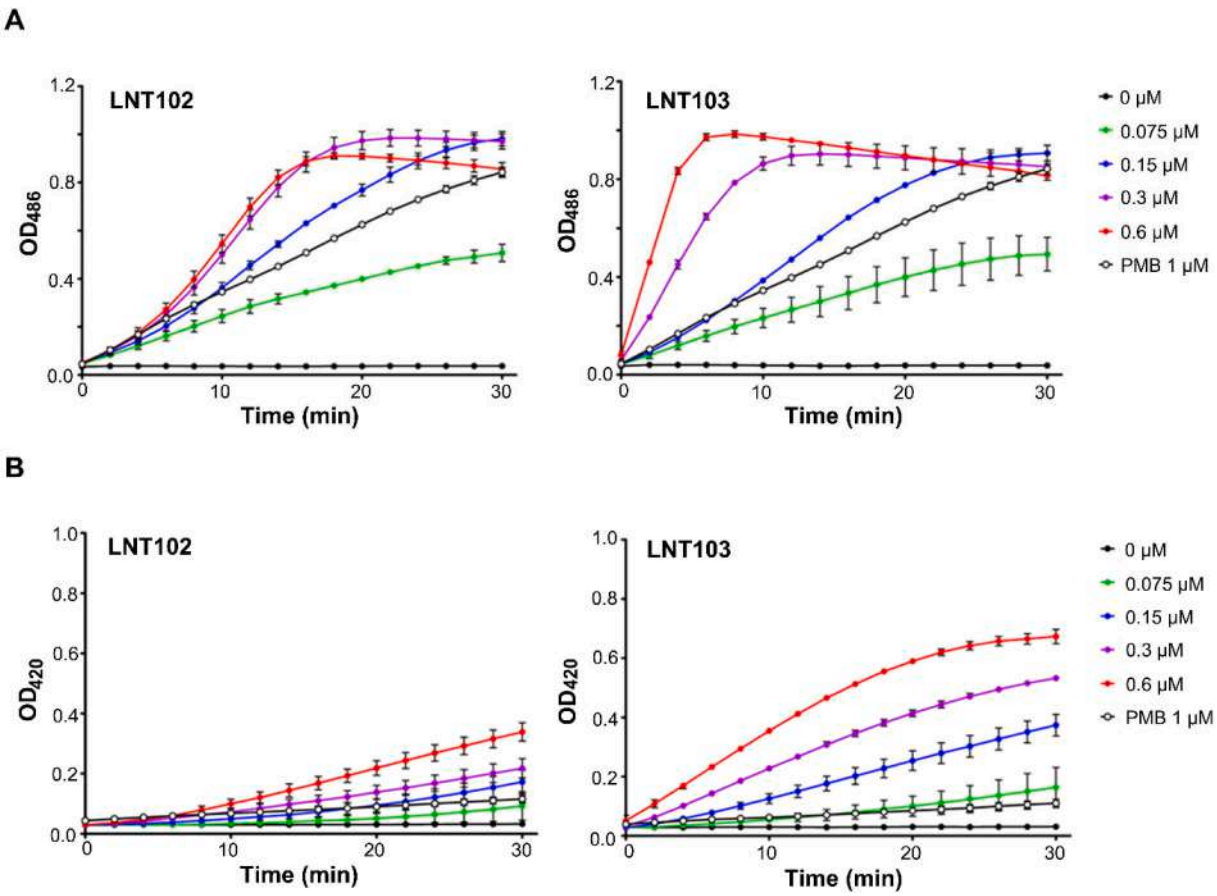

Fig. 4. Cecropin A fusion facilitated the disruption of the double-membraned envelope
Escherichia coli ML-35p cells producing periplasmic β-lactamase and cytoplasmic β-galactosidase were treated with different concentrations of LNT102 (left) and LNT103 (right). (A) The permeability of the outer membrane was estimated by adding nitrocefin, which is hydrolyzed by periplasmic β-lactamase and changes colour at 486 nm. (B) Permeability of the inner membrane was measured by adding *o*-nitrophenyl-β-D- galactopyranoside, which displays colorimetric change at 420 nm by cytoplasmic β-galactosidase. Polymyxin B (PMB) was added as a control at 1 μM.

additive effects of meropenem and the endolysin (Table 2) were verified *in vivo*. Next, blood cytokine levels with the same mice model were examined (Fig. 8D). The presence of a high number of bacteria in this model, at concentrations of IL6 (*ca.* 4000 pg/ml) and TNF-α (*ca.* 600 pg/ml) in the serum, led to an instant cytokine storm. Reported serum cytokine levels of BALB/c mice for IL6 and TNF-α were *ca.* 100 pg/ml and 10 pg/ml, respectively [49,50]. Treatment with meropenem did not alter the cytokine level further, but additional treatment with the endolysin increased the level of TNF-α by 30 %.

3.9. Appearance of anti-drug antibodies

Dogs were IV-treated with LNT103 at indicated doses daily for 14 days. Anti-drug (LNT103) antibody was detected in 23 out of 24 dogs when tested at day 15 (Table 3). Only one male dog administered with 10 mg/kg/day remained antibody negative. Whether the antibody had a neutralizing activity was not determined.

4. Discussion

In the majority of cases, recombinant endolysins derived from phages targeting Gram-negative bacteria exhibit no antibacterial function when applied externally. In addition, it has been seen that the addition of recombinant holins does not improve antibacterial function [51,52].

By contrast, the success of the engineered endolysin LNT103 can be seen to be the result of three important factors. Firstly, the native endolysin from phage PBPA90 (LNT101), targeting *P. aeruginosa*, has a rare intrinsic antibacterial activity. Secondly, site-directed mutagenesis of the 15 amino acids appears to lead to an increase in antibacterial efficacy. Thirdly, fusion with cecropin A, which is a 37-residue antimicrobial peptide (AMP), presumably facilitates further membrane permeabilization [53,54]. Cecropin A and the endolysin may, in close proximity, exert maximal antibacterial activity as a single molecule rather than two separate ones.

Colistin is an antimicrobial peptide in clinical use. When colistin is used in combination with LNT103, there are two different membrane permeabilizers (colistin and cecropin A), and one native endolysin with additional membrane-permeabilizing ability, all working together and leading to a synergistic effect. Unfortunately, colistin use is associated with nephrotoxicity and neurotoxicity, which may limit its general use [55]. However, as the development of carbapenem resistant *Acinetobacter* (CRAB) is rapidly taking place [56], the additive effect of the endolysin LNT103 with meropenem demonstrated *in vitro* and *in vivo* in this work could be a more clinically relevant solution. Membrane permeabilization and the breaking down of the cell wall by LNT103 may facilitate the entry of meropenem, leading to an additive effect. This type of clinical synergy has already been previously reported on, where the additive effects of colistin and meropenem were observed, with colistin serving towards membrane permeabilization [57].

Many endolysins reported on to date, whether derived from phages infecting *A. baumannii* or from phages infecting various other pathogens, have been found to be effective against *A. baumannii*. For example, LNT101 (LysPA90) in this study was originally derived from a phage

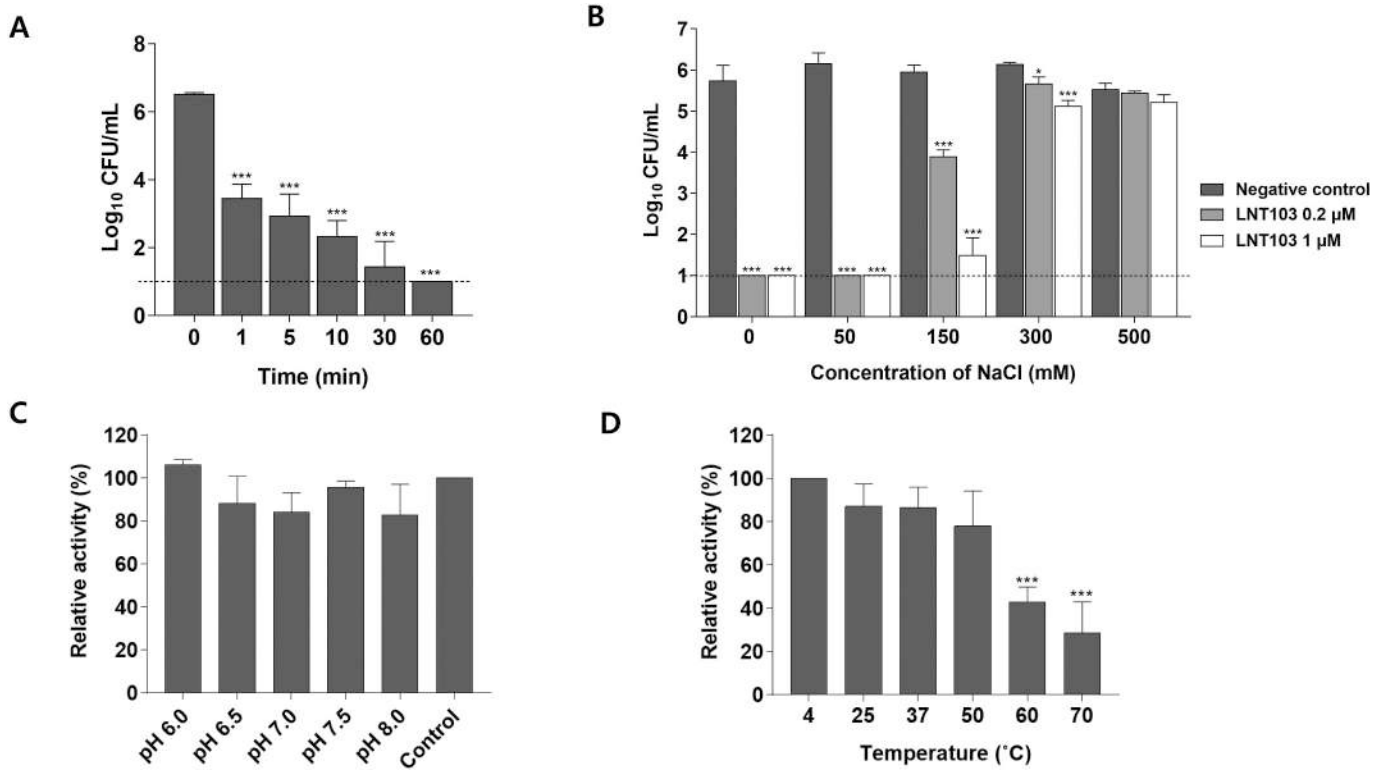

Fig. 5. Comparison of antibacterial activity in various biochemical conditions.
(A) The bacterial count at 0 to 60 min after treatment with the endolysin at 0.2 μM. (B) The bacterial count after treatment with the endolysin at indicated concentrations in the presence of 0 to 500 mM NaCl for 60 min. (C) The bacterial count after treatment with the endolysin at 0.5 μM for 60 min at indicated pHs. (D) The bacterial count after treatment with the endolysin at 0.5 μM for 60 min at indicated temperatures. *A. baumannii* 19,606 was used for the experiments. Each experiment was performed in triplicate.

infecting *P. aeruginosa*, and was seen to be more effective against *A. baumannii* than other Gram-negative pathogens. Another example is endolysin Ts2631, originating from a phage infecting *Thermus scotoductos* [15]. The latter endolysin was observed as being more effective against *A. baumannii* than other Gram-negative pathogens including *P. aeruginosa, E. coli*, and *K. pneumoniae*. The endolysin LysSS originating from a *Salmonella enterica* phage was also more effective against *A. baumannii* than *S. enterica* [16]. Again, we speculate that the particular composition of core oligosaccharide and lipid A in *A. baumannii* could be the determining factor for the higher efficacy of the endolysins against this bacterium, and a further study would be needed.

As presented in the introduction to this study, many previous studies have shown enhanced antimicrobial efficacies of various endolysins with fusion to cecropin A against *A. baumannii*. This was also seen to be the case for endolysin LNT103 in this present study. In the case of *E. coli*, a mutant defective in the core oligosaccharide was observed as being less susceptible to the cecropin-fused endolysin, LNT113, and another mutant with a compromised lipid A construction, which was more vulnerable to it [23]. We can perhaps assume that the compositions of core oligosaccharide and lipid A in *A. baumannii* are the determining factors for the higher efficacy of cecropin A-fused endolysins.

In a series of investigations by our group, the same native endolysin, LNT101 (LysPA90), derived from phage PBPA90, was observed to have been fused to either a cell penetrating peptide DS4.3 [58], an antimicrobial peptide thanatin [59], or, in the present study, the antimicrobial peptide cecropin A. Antimicrobial efficacies *in vitro* were demonstrated and all three exhibited a *ca.* 5-log reduction in CFU counts of *A. baumannii* at 0.2 or 0.25 μM. DS4.3-fused endolysin's *in vivo* efficacy was demonstrated only in a *Galleria mellonella* infection model. In a mouse infection model, the thanatin-fused endolysin assisted, to a limited extent, in the prolonged survival of mice. In the same experiment, the engineered endolysin was intraperitoneally administered following an intraperitoneal infection by *A. baumannii*, in which route bacterial clearance *in situ* occurred. Administered endolysin promptly eliminated the bacteria at the site of injection, limiting the systemic distribution of the bacteria. Accordingly, this may have led to the lowered proinflammatory cytokine levels observed in the study. However, the infection and treatment method used was of limited clinical relevance. On the other hand, the present study utilized intraperitoneal infection followed by intravenous administration of the endolysin, excluding the possibility of intraperitoneal clearance of bacteria *in situ*, allowing for more systemic distribution of the infected bacteria, and exposing the endolysin directly in blood.

Nevertheless, the findings of *in vivo* efficacy in this present report can be translated into clinical investigations with certain limitations, since mice infection models of bacteremia cannot precisely mimic clinical bacteremia [60]. For example, the induction of neutropenia was required to establish bacteremia in mice since *A. baumannii* is an opportunistic pathogen. Even in neutropenic mice, a bolus injection of a large dose of bacteria was needed to overcome host defenses, which does not mimic human bacteremia where a steady stream of bacteria is present. Consequently, the presence of such large numbers of bacteria promptly elicited cytokine storms, possibly leading to prompt organ failure. The intravenous administration method also needs to be considered. Considering the remarkably fast action and relatively short half-life of the endolysin, a slow infusion method would increase the antibacterial efficacy. However, it is technically hard to implement this in mice models. If one can overcome the hurdles of there being up to 1000 times the number of bacteria in the blood for the mice model than for clinical patients, with the resulting cytokine storms, and the less than favorable administration method of IV bolus, higher survival rates than those reported in this study could be expected.

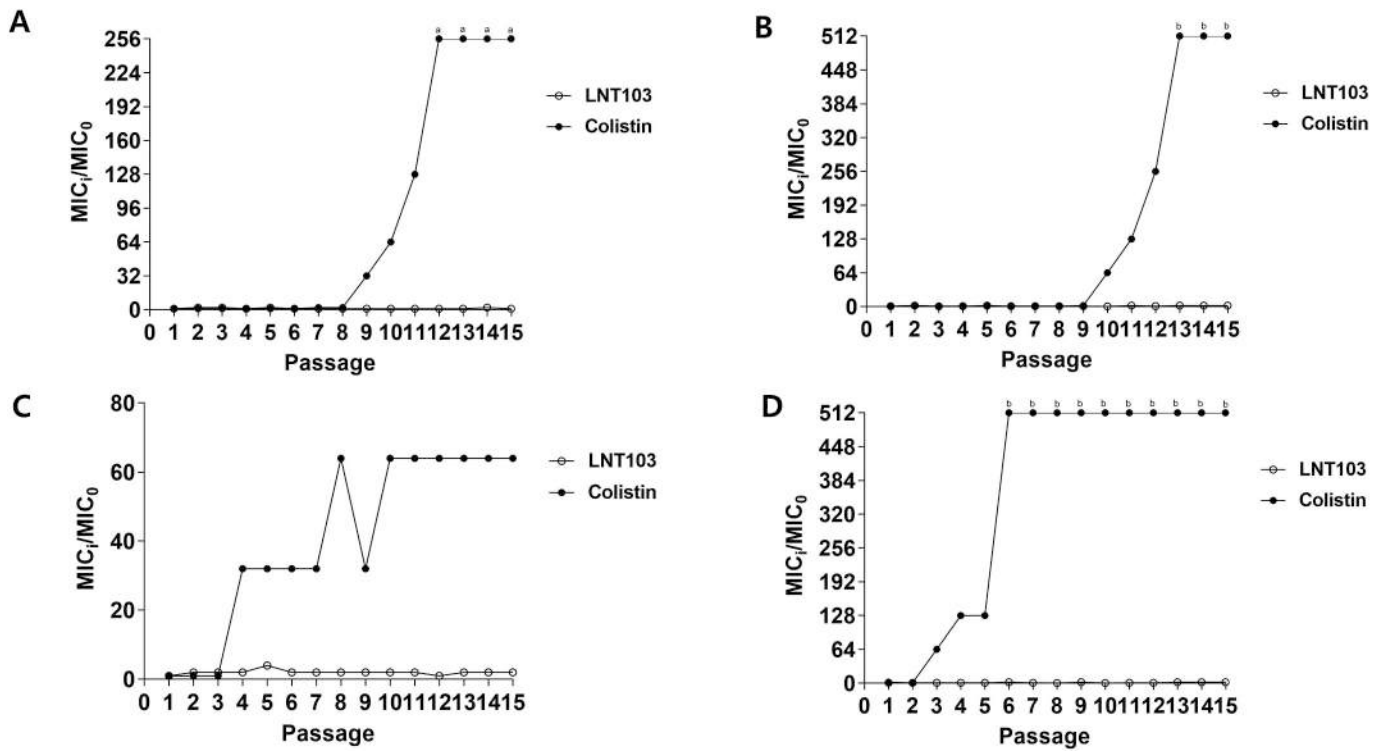

Fig. 6. Bacterial resistance development against LNT103 or colistin
Various strains of *A. baumannii* were serially transferred to new culture medium in the presence of subinhibitory concentrations of LNT103 or colistin for over 15 passages. (A) *A. baumannii* ATCC 19606, (B) *A. baumannii* CCARM 12171, (C) *A. baumannii* CCARM 12065, and (D) *A. baumannii* TA5(26) clinical strains are shown. MIC_0; initial MIC, MIC_i; MIC at the indicated passage. a and b indicate the values were beyond measurement. a; ≥ 256 folds, b; ≥ 512 folds.

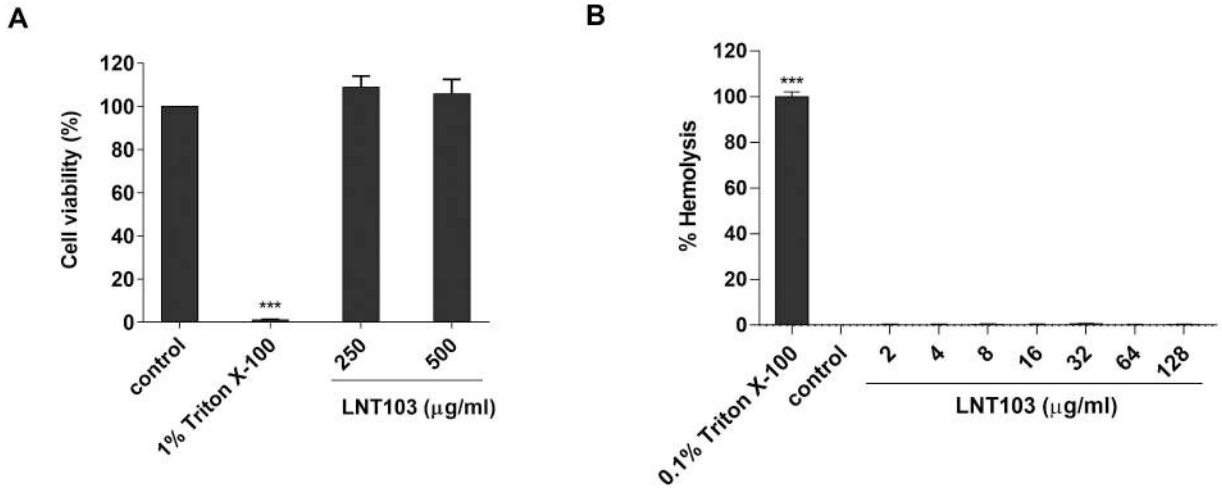

Fig. 7. Cytotoxicity and hemolytic activity assessment of LNT103 *in vitro*
(A) WST-1 cytotoxicity assay of LNT103 in Huh7 cells. Huh7 cells were incubated with indicated concentrations of LNT103 for 48 h. Triton X-100 was used as a cytotoxicity control reagent. (B) Hemolytic activity of LNT103. RBCs were incubated with PBS buffer (control) or LNT103 at indicated concentrations at 37 °C for 1 h, and hemolysis was determined by measuring the absorbance of the supernatant at 570 nm. 0.1 % of Triton X-100 was used as a hemolytic control reagent inducing 100 % hemolysis. Experiments were performed at least three times. Student's *t*-test was performed against each control and the *p* values are shown above each bar (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The endolysin LNT103 has clearly demonstrated antibacterial efficacy against *A. baumannii* in the present mice models. Notably, it was very effective in combination with meropenem when combating the meropenem-resistant strain of *A. baumannii*. As carbapenem-resistant *A. baumannii* (CRAB) are increasingly being encountered, especially in intensive care units (ICUs), resensitization of the resistant pathogens to carbapenem antibiotics using the endolysin could be a viable method for combating the deadly bacteria.

Daily administration of the endolysin for two weeks led to the appearance of anti-drug antibodies in most dogs tested. As the endolysin was a foreign material, it is expected to elicit immunogenic reactions naturally from the animals. However, the present study did not evaluate the neutralizing activity of the antibodies. Considering the fast action of an endolysin seen in the mice models in this study, a prolonged

Table 2
Combination effect of the endolysin with various antibiotics as determined by checkerboard assays.

Antibiotics	$FIC_{antibiotic}$	FIC_{LNT103}	FIC_{index}	Interpretation
Colistin	0.250	0.250	0.500	Synergistic
Meropenem	0.208	0.500	0.708	Additive
Imipenem	0.250	0.500	0.750	Additive
Ceftazidime	0.417	0.500	0.917	Additive
Tigecycline	0.667	0.667	1.334	Indifferent

FIC index <05; synergy.
FIC index 0.5–1.0; additive.
FIC index >1.0; indifferent.

administration (*e.g.* 14 days) of the endolysin may not be needed in clinical situations. Moreover, a shorter period of administration may not lead to the appearance of anti-drug antibodies. A further study about immunogenic reactions against the endolysin is thus anticipated and awaited.

Given the broad spectrum of the endolysin, one might worry about potential side effects on microbiota. The rationale for using this endolysin is as follows; 1) *In vivo* evaluation in this report was made for bacteremia models, and no microbiota would have been present for the serum or organs. 2) In case the endolysin was used for gut infection, alteration of microbiota would be conceivable based on the wide range of Gram-negative microbes affected by the endolysin. But, considering the enzymatic nature of the endolysin, gut infection where countless impurities for enzyme reaction are present, would be the last indication for a targeted endolysin. 3) Even if alteration of microbiota by endolysins occurred, it could be rectified once the treatment was terminated. Cures for a life-threatening infection by pathogens would be a priority over and above that of conserving normal microbiota.

5. Conclusions

In summary, the engineered endolysin LNT103 has the potential to serve as a new modality for antibiotics, making them effective against multidrug-resistant pathogens without allowing for the development of resistance *in vitro*. Moreover, the synergistic or additive effects with currently prescribed antibiotics shown *in vitro* and *in vivo* suggest that the endolysin would be a clinically relevant candidate as a novel antibiotic.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.140463.

Sequence information

The sequence for the endolysin from bacteriophage PBPA90 was deposited in GenBank under the accession number of MW815133.

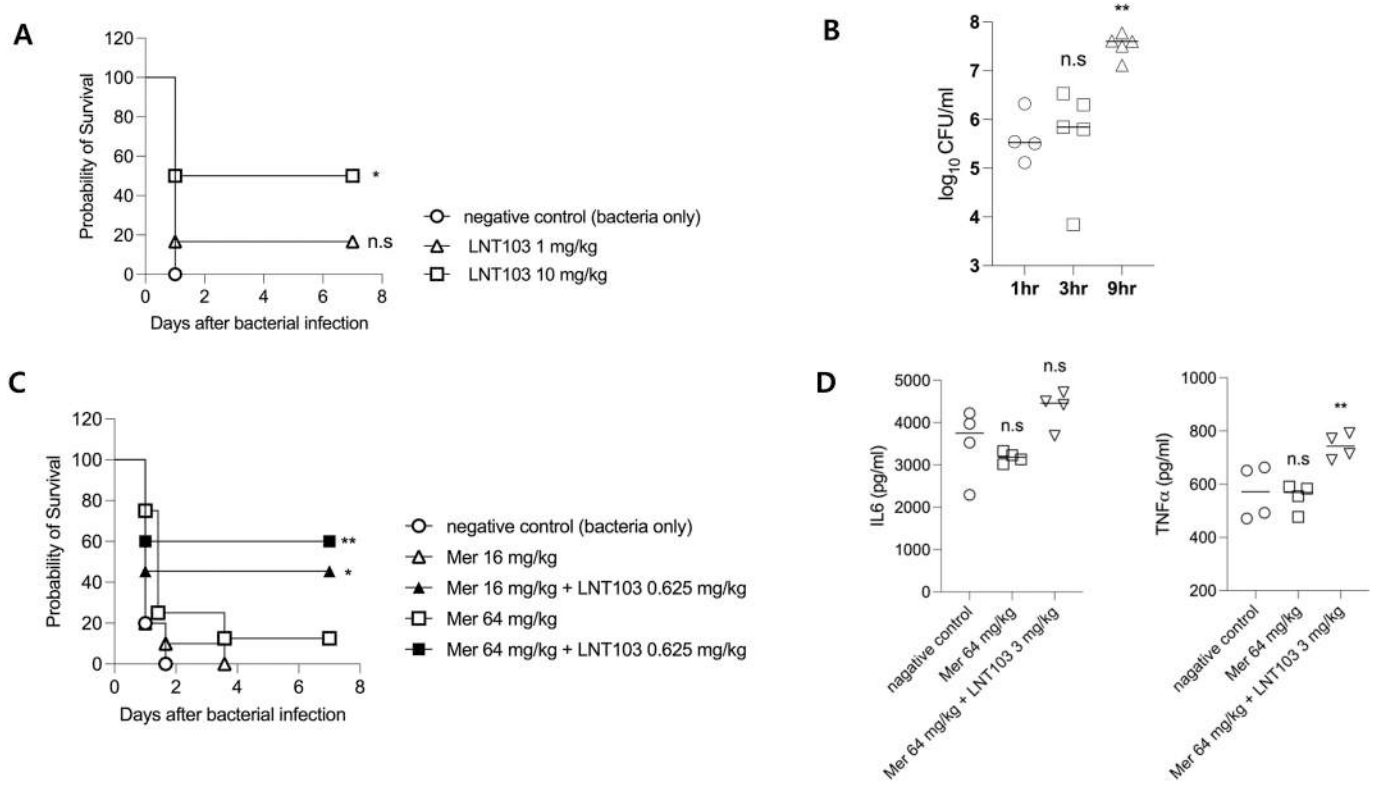

Fig. 8. *In vivo* efficacy of LNT103
(A) Mice pre-treated with cyclophosphamide to induce neutropenia and IP-infected with 10^8 PFU of *A. baumannii* ATCC19606. At 1 h post infection, the mice were IV-treated with endolysin LNT103 at 1 or 10 mg/kg. Each group contained five mice. (B) Mice pre-treated with cyclophosphamide to induce neutropenia and IP-infected with 10^8 PFU of *A. baumannii* ATCC19606. At 1, 3, or 9 h post infection, the mice were sacrificed and the titer of *A. baumannii* was measured from the blood. 4 mice were used for 1 h, and 5 mice were used for 3 and 9 h. (C) Mice pre-treated with cyclophosphamide to induce neutropenia and IP-infected with 10^7 PFU of *A. baumannii* CCARM12233 which is resistant to carbapenem antibiotics. At 1 h post infection, mice were SC-treated with meropenem at 16 or 64 mg/kg, with or without combination IV-treatment with endolysin LNT103 at 0.625 mg/kg. Each group contained 10 mice. (D) Mice were pre-treated with cyclophosphamide to induce neutropenia and IP-infected with 10^7 PFU of *A. baumannii* CCARM12233 resistant to carbapenem antibiotics. At 1 h post infection, mice were either SC-treated with meropenem at 64 mg/kg alone, or in combination of IV-treatment with endolysin LNT103 at 3 mg/kg. The mice were sacrificed at 16 h post infection and serum cytokine levels of IL6 and TNF-α were measured by ELISA. 4 mice were used for each group. Statistical calculations for mice survival rates were performed by the Kaplan-Meier method with log-rank (Mantel-Cox) tests in Prism version 9 (GraphPad, Dotmatics) (* $p < 0.05$, ** $p < 0.01$).

Table 3
Appearance of anti-drug antibodies in dogs.

Group (dose)	Sex	Animal no.	Sampling point	Evaluation
1 (control)	Male	1101	D0	−
			D15	−
		1102	D0	−
			D15	−
		1103	D0	−
			D15	−
	Female	2101	D0	−
			D15	−
		2102	D0	−
			D15	−
		2103	D0	−
			D15	−
2 (3 mg/kg/day)	Male	1201	D0	−
			D15	+
		1202	D0	−
			D15	+
		1203	D0	−
			D15	+
	Female	2201	D0	−
			D15	+
		2202	D0	−
			D15	+
		2203	D0	−
			D15	+
3 (10 mg/kg/day)	Male	1301	D0	−
			D15	+
		1302	D0	−
			D15	−
		1303	D0	−
			D15	+
	Female	2301	D0	−
			D15	+
		2302	D0	−
			D15	+
		2303	D0	−
			D15	+

CRediT authorship contribution statement

Hye-Won Hong: Investigation. **Jaeyeon Jang:** Investigation. **Young Deuk Kim:** Investigation. **Tae-Hwan Jeong:** Investigation. **Dogeun Lee:** Investigation. **Kyungah Park:** Investigation. **Min Soo Kim:** Formal analysis, Conceptualization. **In-Soo Yoon:** Formal analysis, Conceptualization. **Miryoung Song:** Formal analysis, Conceptualization. **Min-Duk Seo:** Formal analysis. **Hyunjin Yoon:** Formal analysis, Conceptualization. **Daejin Lim:** Formal analysis, Conceptualization. **Heejoon Myung:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics approval

All animal procedures were conducted in accordance with the relevant guidelines for the care and use of laboratory animals and approved by the Animal Ethics Committee of Kangwon National University (KW-231019-4).

Funding

This work was supported by the Bio-industry Technology Development Project (grant number 20018377) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea), the Korea Health Industry Development Institute (KHIDI, Korea) (grant number HI21C2447), and the HUFS Research Fund of 2024.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hye-Won Hong reports financial support was provided by LyseNTech. Jaeyeon Jang reports financial support was provided by LyseNTech. Young Deuk Kim reports financial support was provided by LyseNTech. Tae-Hwan Jeong reports financial support was provided by LyseNTech. Min Soo Kim reports financial support was provided by LyseNTech. Heejoon Myung reports financial support was provided by LyseNTech. Heejoon Myung has patent POLYPEPTIDE, FUSION POLYPEPTIDE, AND ANTIBIOTIC AGAINST GRAM-NEGATIVE BACTERIA COMPRISING SAME issued to LyseNTech. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

H-WH, JJ, YDK, THJ, MK, and HM are employed by LyseNTech. Co. Ltd. (Korea).

Data availability

The DNA sequence of the native endolysin from phage PBPA90 was deposited in GenBank under the accession number MW815133. The source data are available from the corresponding authors on reasonable request.

References

[1] L. Grabowski, K. Lepek, M. Stasilojc, et al., Bacteriophage-encoded enzymes destroying bacterial cell membranes and walls, and their potential use as antimicrobial agents, Microbiol. Res. 248 (2021) 126746–126765, https://doi.org/10.1016/j.micres.2021.126746.
[2] J. Cahill1, R. Young., Phage lysis: multiple genes for multiple barriers, Adv. Virus Res. 103 (2019) 33–70, https://doi.org/10.1016/bs.aivir.2018.09.003.
[3] R. Vázquez, E. García, P. García, Phage Lysins for fighting bacterial respiratory infections: a new generation of antimicrobials, Front. Immunol. 9 (2018) 2252, https://doi.org/10.3389/fimmu.2018.02252.
[4] V.S. Gondil, K. Harjai, S. Chhibber, Endolysins as emerging alternative therapeutic agents to counter drug-resistant infections, Int. J. Antimicrobial. Agents 55 (2020) 105844, https://doi.org/10.1016/j.ijantimicag.2019.11.001.
[5] F. Abdelrahman, M. Easwaran, O.I. Daramola, et al., Phage-Encoded Endolysins. Antibiotics 10 (2021) 124–152, https://doi.org/10.3390/antibiotics10020124.
[6] A. Bhagwat, M. Mixon, C.H. Collins, et al., Opportunities for broadening the application of cell wall lytic enzymes, Applied Microbiol. Biotechnol. 104 (2020) 9019–9040, https://doi.org/10.1007/s00253-020-10862-y.
[7] Clinical trial identifier NCT04160468, www.clinicaltrials.gov.
[8] Clinical trial identifier NCT01746654, www.clinicaltrials.gov.
[9] Clinical trial identifier NCT03089697, www.clinicaltrials.gov.
[10] W.C.B. Laia, X. Chen, M.K.Y. Ho, et al., Bacteriophage-derived endolysins to target gram-negative bacteria, Int. J. Pharm. 589 (2020) 119833–119849, https://doi.org/10.1016/j.ijpharm.2020.119833.
[11] M. Walmagh, B. Boczkowska, B. Grymonprez, et al., Characterization of five novel endolysins from gram-negative infecting bacteriophages, Appl. Microbiol. Biotechnol. 97 (2013) 4369–4375, https://doi.org/10.1007/s00253-012-4294-7.
[12] B. Maciejewska, B. Roszniowski, A. Espaillat, et al., Klebsiella phages representing a novel clade of viruses with an unknown DNA modification and biotechnologically interesting enzymes, Appl. Microbiol. Biotechnol. 101 (2017) 673–684, https://doi.org/10.1007/s00253-016-7928-3.
[13] M. Guo, C. Feng, J. Ren, et al., A novel antimicrobial Endolysin, LysPA26, against *Pseudomonas aeruginosa*, Front. Microbiol. 8 (2017) 293, https://doi.org/10.3389/fmicb.2017.00293.
[14] M. Wu, K. Hu, Y. Xie, et al., A novel phage PD-6A3, and its Endolysin Ply6A3, with extended lytic activity against *Acinetobacter baumannii*, Front. Microbiol. 9 (2019) 3302, https://doi.org/10.3389/fmicb.2018.03302.
[15] M. Plotka, M. Kapusta, S. Dorawa, et al., Ts2631 Endolysin from the Extremophilic *Thermus scotoductus* bacteriophage vB_Tsc2631 as an antimicrobial agent against gram-negative multidrug-resistant Bacteria, Viruses 11 (2019) 657–671, https://doi.org/10.3390/v11070657.
[16] S. Kim, D-W. Lee, J-S. Jin et al. Antimicrobial activity of LysSS, a novel phage endolysin, against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. J. Global Antimicrob. Resistance 22 (2020) 32–39 doi:https://doi.org/10.1016/j.jgar.2020.01.005.
[17] F.M. Khan, V.S. Gondil, C. Li, et al., A novel *Acinetobacter baumannii* bacteriophage Endolysin LysAB54 with high antibacterial activity against multiple gram-negative microbes, Front. Cell. Infect. Microbiol. 11 (2020) 637313, https://doi.org/10.3389/fcimb.2021.637313.
[18] Y. Briers, M. Walmagh, V. Van Puyenbroeck, et al., Engineered endolysin-based "Artilysins" to combat multidrug-resistant gram-negative pathogens, mBio 5 (2014), https://doi.org/10.1128/mBio.01379-14 e01379–14.
[19] V. Defraine, J. Schuermans, B. Grymonprez, et al., Efficacy of Artilysin Art-175 against resistant and persistent *Acinetobacter baumannii*, Antimicrob. Agents Chemother. 60 (2016) 3480–3488, https://doi.org/10.1128/AAC.00285-16.

[20] K. Abdelkader, D. Gutierrez, H. Tames-Caunedo, P. Ruas-Madiedo, A. Safaan, A. S. Khairalla, et al., Engineering a lysin with intrinsic antibacterial activity (LysMK34) with cecropin a enhances its antibacterial properties against *Acinetobacter baumannii*, Appl. Environ. Microbiol. 88 (2022) e0151521, https://doi.org/10.1128/AEM.01515-21.
[21] H-W Hong, Y.D Kim, J. Jang, J et al. Combination effect of engineered Endolysin EC340 with antibiotics. Front. Microbiol. 13(2022):821936 doi: https://doi.org/10.3389/fmicb.2022.821936.
[22] H. Gerstmans, L. Duyvejonck, R. Vázquez, et al., Distinct mode of action of a highly stable, engineered phage lysin killing gram-negative bacteria, Microbiol Spectr 11 (2023) e0181323, https://doi.org/10.1128/spectrum.01813-23.
[23] J. Cho, H.-W. Hong, K. Park, et al., Unveiling the mechanism of bactericidal activity of a cecropin A-fused endolysin LNT113, Int. J. Biol. Macromol. 260 (2024) 129493.
[24] J. Lim, J. Hong J, Y. Jung et al. Bactericidal Effect of Cecropin A Fused Endolysin on Drug-Resistant Gram-Negative Pathogens. *J Microbiol Biotechnol* 32(2022) 816–823.
[25] H. Yang, M. Wang, J. Yu, H. Wei, Antibacterial activity of a novel peptide-modified Lysin against Acinetobacter baumannii and Pseudomonas aeruginosa, Front. Microbiol. 6 (2015) 1471, https://doi.org/10.3389/fmicb.2015.01471.
[26] X. Chen, M. Liu, P. Zhang, et al., Membrane-permeable antibacterial enzyme against multidrug-resistant Acinetobacter baumannii, ACS Infect Dis 7 (2021) 2192–2204, https://doi.org/10.1021/acsinfecdis.1c00222.
[27] N.P. Antonova, I.V. Grigoriev, A.M. Lendel, et al., Engineering of recombinant Endolysin LysSi3 to increase its antibacterial properties, Appl. Biochem. Microbiol. 60 (2024) 802–811, https://doi.org/10.1134/S000368382460492X.
[28] S-Y. Peng, R-I. You, M-J. Lai et al. Highly potent antimicrobial modified peptides derived from the *Acinetobacter baumannii* phage endolysin LysAB2. Sci. Rep. 7 (2017) 11477 doi:https://doi.org/10.1038/s41598-017-11832-7.
[29] S.M. Son, J. Kim, S. Ryu, Development of sensitizer peptide-fused endolysin Lys1S-L9P acting against multidrug-resistant gram-negative bacteria, Front. Microbiol. 14 (2023) 1296796, https://doi.org/10.3389/fmicb.2023.1296796.
[30] H.M. Sisson, S.A. Jackson, R.D. Fagerlund, S.L. Warring, P.C. Fineran, Gram-negative endolysins: overcoming the outer membrane obstacle, Curr. Opin. Microbiol. 78 (2024) 102433, https://doi.org/10.1016/j.mib.2024.102433.
[31] J.V. Carratalá, A. Arís, E. Garcia-Fruitós, N. Ferrer-Miralles, Design strategies for positively charged endolysins: insights into Artilysin development, Biotechnol. Adv. 69 (2023) 108250, https://doi.org/10.1016/j.biotechadv.2023.108250.
[32] R.I. Lehrer, A. Barton, K.A. Daher, et al., Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity, J. Clin. Invest. 84 (1989) 553–561, https://doi.org/10.1172/JCI114198.
[33] R.I. Lehrer, A. Barton, T. Ganz, Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in *E. Coli* by multiple-wavelength spectrophotometry, J. Immunol. Methods 108 (1988) 153–158, https://doi.org/10.1016/0022-1759(88)90414-0.
[34] C.R. Bonapace, J.A. Bosso, L.V. Friedrich, R.L. White, Comparison of methods of interpretation of checkerboard synergy testing, Diagn. Microbiol. Infect. Dis. 44 (2002) 363–366, https://doi.org/10.1016/s0732-8893(02)00473-x.
[35] R. Thummeepak, T. Kitti, D. Kunthalert, S. Sitthisak, Enhanced antibacterial activity of Acinetobacter baumannii bacteriophage ØABP-01 Endolysin (LysABP-01) in combination with Colistin, Front. Microbiol. 7 (2016) 1402, https://doi.org/10.3389/fmicb.2016.01402.
[36] Y. Briers, M. Walmagh, B. Grymonprez, et al., Art-175 is a highly efficient antibacterial against multidrug-resistant strains and Persisters of Pseudomonas aeruginosa, Antimicrob. Agents Chemother. 58 (2014) 3774–3784, https://doi.org/10.1128/AAC.02668-14.
[37] I. Czink, A. Koncz, S. Molnar et al. Neutropenia induced by cyclophosphamide. Mouse model. *Pharmacol Res* 25(1992) Supplement 2, 296.
[38] A.F. Zuluaga, B.E. Salazar, C.A. Rodriguez, et al., Neutropenia induced in outbred mice by a simplified low-dose cyclophosphamide regimen: characterization and applicability to diverse experimental models of infectious diseases, BMC Infect. Dis. 6 (2006) 55.
[39] M. Russo, N. Panini, P. Fabbrizio, et al., Chemotherapy-induced neutropenia elicits metastasis formation in mice by promoting proliferation of disseminated tumor cells, OncoImmunol 12 (2023) 2239035.
[40] R. Vázquez, E. García, P. García, Sequence-function relationships in phage-encoded bacterial Cell Wall lytic enzymes and their implications for phage-derived product design, J. Virol. 95 (2021) e0032121, https://doi.org/10.1128/JVI.00321-21.
[41] D. Gutiérrez, Y. Briers, Lysins breaking down the walls of gram-negative Bacteria, no longer a no-go, Curr. Opin. Biotechnol. 68 (2021) 15–22, https://doi.org/10.1016/j.copbio.2020.08.014.
[42] J. Kim, B.K. Hwang, H. Choi, et al., Characterization of mcr-1-harboring plasmids from Pan drug-resistant Escherichia coli strains isolated from retail raw chicken in South Korea, Microorganisms 7 (2019) 344, https://doi.org/10.3390/microorganisms7090344.
[43] B. Li, F. Yin, X. Zhao, Y. Guo, et al., Colistin resistance gene *mcr-1* mediates cell permeability and resistance to hydrophobic antibiotics, Front. Microbiol. 10 (2020) 3015, https://doi.org/10.3389/fmicb.2019.03015.
[44] M.M. Zafer, H.A. El-Mahallawy, A. Abdulhak, et al., Emergence of colistin resistance in multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from cancer patients, Ann. Clin. Microbiol. Antimicrob. 18 (2019) 40, https://doi.org/10.1186/s12941-019-0339-4.
[45] H. Zhang, M. Hou, Y. Xu, et al., Action and mechanism of the colistin resistance enzyme MCR-4, Commun. Biol. 2 (2019) 36, https://doi.org/10.1038/s42003-018-0278-1.
[46] E. Casarotta, E. Bottari, S. Vannicola, et al., Antibiotic treatment of *Acinetobacter baumannii* superinfection in patients with SARS-CoV-2 infection admitted to intensive care unit: an observational retrospective study, Front. Med. 9 (2022) 910031, https://doi.org/10.3389/fmed.2022.910031.
[47] Z.A. Kanafani, and S.S. Kanj. Acinetobacter infection: Treatment and prevention. https://www.uptodate.com/contents/acinetobacter-infection-treatment-and-prevention.
[48] O. Opota, A. Croxatto, G. Prod'hom, G. Greub, Blood culture-based diagnosis of bacteremia: state of the art, Clin. Microbiol. Infect. 21 (2015) 313–322, https://doi.org/10.1016/j.cmi.2015.01.003.
[49] Y. Li, L. Wang, L.Y. Zhang, et al., Serum cytokine modulation after *staphylococcus hyicus* infection in BALB/c mice, Genet. Mol. Res. 14 (2015) 16682–16693.
[50] B.L. Ferreira, E.R. Ferreira, M.V. de Brito, et al., BALB/c and C57BL/6 mice cytokine responses to Trypanosoma cruzi infection are independent of parasite strain infectivity, Front. Microbiol. 9 (2018) 553.
[51] I.N. Wang, D.L. Smith, R. Young, Holins: the protein clocks of bacteriophage infections, Annu. Rev. Microbiol. 54 (2000) 799–825, https://doi.org/10.1146/annurev.micro.54.1.799.
[52] M.J. Catalão, F. Gil, J. Moniz-Pereira, et al., Diversity in bacterial lysis systems: bacteriophages show the way, FEMS Microbiol. Rev. 37 (2013) 554–571, https://doi.org/10.1111/1574-6976.12006.
[53] L. Silvestro, J.N. Weiser, P.H. Axelsen, Antibacterial and Antimembrane activities of Cecropin a in *Escherichia coli*, Antimicrob. Agents Chemother. 44 (2000) 602–607, https://doi.org/10.1128/aac.44.3.602-607.2000.
[54] Q. Wu, J. Patočka, K. Kuča, Insect Antimicrobial Peptides, a Mini Review, Toxins 10 (2018) 461, https://doi.org/10.3390/toxins10110461.
[55] I. Kilic1, Y. Ayar, I. Ceylan et al. Nephrotoxicity caused by colistin use in ICU: a single Centre experience BMC Nephrol. 24 (2023) 302 doi:https://doi.org/10.1186/s12882-023-03334-8.
[56] Y. Jiang, Y. Ding, Y. Wei, et al., Carbapenem-resistant *Acinetobacter baumannii*: a challenge in the intensive care unit, Front. Microbiol. 13 (2022) 1045206, https://doi.org/10.3389/fmicb.2022.1045206.
[57] W. Katip, S. Uitrakul, P. Oberdorfer, A comparison of Colistin versus Colistin plus Meropenem for the treatment of Carbapenem- resistant Acinetobacter baumannii in critically ill patients: a propensity score-matched analysis, Antibiotics 9 (2020) 647, https://doi.org/10.3390/antibiotics9100647.
[58] J. Lim, J. Jang, H. Myung, M. Song, Eradication of drug-resistant *Acinetobacter baumannii* by cell-penetrating peptide fused endolysin, J. Microbiol. 60 (2022) 859–866.
[59] J. Lim, H. Myung, D. Lim, M. Song, Antimicrobial peptide thanatin fused endolysin PA90 (Tha-PA90) for the control of *Acinetobacter baumannii* infection in mouse model, J. Biomed. Sci. 31 (2024) 36.
[60] A.J. Lewis, C.W. Seymour, M.R. Rosengart, Current murine models of sepsis, Surg. Infect. (Larchmt.) 17 (2016) 385–393, https://doi.org/10.1089/sur.2016.021.