ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

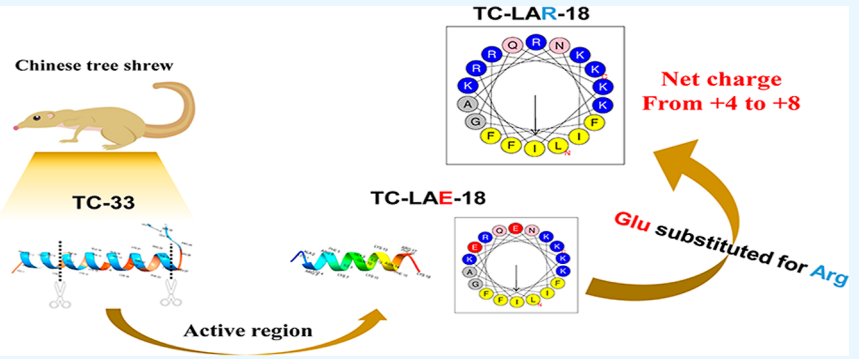
Novel Tree Shrew-Derived Antimicrobial Peptide with Broad-Spectrum Antibacterial Activity

Lin Luo,[#] Ying Cai,[#] Yunhan Su,[#] Chenxi Li, Gengzhou Tian, Xingyu Wang, Zhongxiang Wu, Wenlin Chen,[*] Tianyu Zhang,[*] and Zhiye Zhang[*]

Cite This: *ACS Omega* 2024, 9, 45279−45288

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



ABSTRACT: The number of cationic residues and net charge are critical for the activity of antimicrobial peptides (AMPs) due to their role in facilitating initial electrostatic interactions with negatively charged bacterial membranes. A cathelicidin AMP (TC-33) has been identified from the Chinese tree shrew in our previous work, which exhibited weak antimicrobial activity, likely due to its moderately cationic nature. In the current study, based on TC-33, we designed a novel AMP by peptide truncation and Glu substitutions to increase its net cationic charge from +4 to +8. The resulting peptide, TC-LAR-18, showed 4−128-fold enhanced antimicrobial activity relative to TC-33 without causing hemolysis and cytotoxicity within 100 μg/mL. TC-LAR-18 effectively eliminated both planktonic and biofilm-associated bacteria, demonstrating rapid bactericidal effects due to its ability to quickly penetrate and disrupt bacterial cell membranes with a low propensity to induce resistance. Notably, TC-LAR-18 provided substantial protection against skin bacterial infection in mice, underscoring its therapeutic potential. These findings not only highlight the importance of positively charged residues for the antibacterial activity of AMPs but also present a useful drug candidate for combating multidrug-resistant bacteria.

1. INTRODUCTION

The discovery of antibiotics promises significant advancements in the treatment of bacterial infections. However, prolonged and excessive use of antibiotics in various fields, including medicine, food, and animal husbandry, has led to the presence of multidrug-resistant bacteria.[1,2] The World Health Organization predicts more than 700,000 people will die each year from drug-resistant bacterial infections.[3,4] Consequently, there is an urgent need to develop antimicrobial agents with new structures and mechanisms of action.

Antimicrobial peptides (AMPs), also known as host defense peptides, are naturally occurring molecules found in bacteria, protists, plants, and animals.[5] They play a key role in invading pathogens due to their unique mechanisms of action. Typically, AMPs possess a positive net charge, allowing them to bind to negatively charged bacterial membranes. They penetrate the bacterial membrane by forming channels or pores in either a "barrel-stave" or "toroidal" arrangement, or by aggregating on the membrane surface to cause lesions without insertion.[6,7] Eventually, the bacteria die due to cell membrane lysis and the leakage of cellular contents. Most natural AMPs (∼74%) originate from animals, especially mammals and insects.[8,9] These AMPs are secreted by their respective cells and are critical elements of the innate immune system.[10] AMPs offer several advantages, including potent, rapid antimicrobial activity, a lower likelihood of developing resistance, and lethality against slow-growing or metabolically inactive microorganisms (e.g., persisters).[11,12] Thus, AMPs hold considerable potential for the development of novel antimicrobial drugs.

Received: July 25, 2024
Revised: October 22, 2024
Accepted: October 24, 2024
Published: October 30, 2024

Cathelicidins are an evolutionarily conserved family of AMPs integral to the vertebrate innate immune system.[13] These peptides demonstrate broad-spectrum antimicrobial efficiency in vitro and in vivo.[14] While AMPs typically possess a positive charge (+1 to +7), short sequences (<50 residues) and an amphiphilic nature,[15] cathelicidins are typically more cationic, with an average charge of +8.13 and an average length of 34.38 residues across all 153 identified cathelicidins.[5] Research has consistently shown a strong correlation between net charge and antimicrobial efficacy,[16,17] with higher cationic charges enhancing attraction to the negatively charged membrane of bacteria, thereby increasing antimicrobial activity. Additionally, most cathelicidins can adopt an amphipathic α-helical structure, characterized by a hydrophilic and hydrophobic face, typically formed by basic and hydrophobic residues, respectively. Cationic residues enable initial binding to negatively charged bacterial membranes, and hydrophobic regions facilitate internalizing and disrupting bacterial membranes.[18,19] Recently, there are 27 AMPs in clinical trials, and nine of them display a structure amphipathic α-helix,[20,21] indicating a structurally ideal template for antimicrobial drug development. Human cathelicidin LL-37 is among the most extensively studied AMPs, generating considerable interest in the design of new peptides.[22] LL-37-derived peptides have shown efficacy in treating infections in animal models[16,23] and chronic otitis media.[16] In our previous work, a cathelicidin AMP was identified from the snake venom of *Bungarus fasciatus* (cathelicidin-BF), which exhibited strong and rapid antimicrobial activity.[24,25] After a decade of research, cathelicidin-BF has been successfully used to treat colpitis and was approved for clinical trials in 2018.[17]

In recent research, a new cathelicidin (TC-33) was also identified from the Chinese tree shrew.[26] Despite possessing nine basic residues, its net charges were largely neutralized by its five acidic Glu residues, leading to a net charge of +4 and relatively low antimicrobial activity.[26] In the present study, we designed a novel AMP based on TC-33 (TC-LAR-18) through peptide truncation and Glu substitutions. Notably, TC-LAR-18 exhibited potent and broad-spectrum antibacterial activity without inducing hemolytic or cytotoxic effects. The antimicrobial effects, mechanisms of action, and therapeutic potential of TC-LAR-18 were thoroughly explored, highlighting its potential as a promising antimicrobial drug candidate for combating multidrug-resistant bacterial pathogens.

2. MATERIALS AND METHODS

2.1. Peptide Synthesis. All peptides were synthesized by GL Biochem Ltd. (Shanghai, China) with purities higher than 98%, as validated by reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry.

2.2. Ethics Approval. All animal experimental environments complied with the relevant Chinese national standards for Experimental Animal Environment and Facilities (GB14925-2010) for barrier environment experimental facilities. The management of animal breeding and experimental operations was in accordance with the Regulations on the Management of Experimental Animals in Yunnan Province and other regulations. All experimental procedures using mice were approved by the Research Ethics Board, Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College (DWSP20240301).

2.3. Bioinformatic Analysis and Structural Modeling. Physicochemical characteristics of all peptides were analyzed by the ExPASy Bioinformatics Resource Portal (http://www.expasy.org/tools/). The helical wheel of the peptides was constructed by HeliQuest (http://heliquest.ipmc.cnrs.fr/). We predicted the peptide 3D structures (the structures were energy minimized and optimized during simulation to finally get the optimal structure) by the PEP-FOLD3 framework (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/#problems).[27] The peptide structures and the surface electrostatic potential of the peptides were visualized and analyzed by PyMOL.

2.4. Antimicrobial Activity In Vitro. Antibacterial activity of the peptides was determined using a microdilution assay as previously described.[28] In addition to standard bacterial strains *Acinetobacter baumannii* ATCC19606, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC8739, and *Staphylococcus aureus* ATCC6538, 26 clinically isolated strains (*A. baumannii* (19110, 10769, and 0357), *P. aeruginosa* (90068, 60357, 52097, and 17068), *E. coli* (0894, 5017, and 1007), *S. aureus* (08310, 15772, 15192, 157752, 11842, 220823, and 15775), and methicillin-resistant *S. aureus* (MRSA) (Z, 11, 12, 21, 22, 41, 42, 51, and 52)) were also used in the antimicrobial assay. Luria−Bertani (LB) medium was widely used for the cultivation of bacteria. According to previous studies from our group[17,29] and others,[30] all bacterial strains were cultured in LB medium.

2.5. Cytotoxic and Hemolytic Activity Assays. The potential cytotoxic and hemolytic activities of the peptide tested were determined according to our previous methods.[29] Briefly, the mammalian cells (human HaCaT keratinocytes and HEK293 embryonic cells) were used and first cultured in 96-well plates (5×10^5 cells/well) with Dulbecco's modified Eagle's medium containing 1% antibiotics and 10% fetal bovine serum at 37 °C in CO_2 (5%). Varied concentrations of peptides (3.125−100 μg/mL) were then added and incubated for 24 h, followed by the addition of CCK-8 agent and incubation at 37 °C for another 2 h according to the manufacturer's instructions (CCK-8, Lot. no. 159077, Med Chem Express). The absorbance at 450 nm was measured with a microplate reader (Epoch, Bio Tek, USA). The percentage of cell viability was calculated relative to the control group without peptide after solution background (without peptide and cells) subtraction.

Freshly obtained mouse red blood cells were used in the hemolytic activity assay. Aliquots (125 μL) of the blood suspension were mixed with an equal volume of peptide solution (1.563−100 μg/mL). Hemoglobin released in the supernatant was then determined at 540 nm with a microplate reader.

2.6. Biofilm Inhibition and Eradication. In brief, *A. baumannii* ATCC19606, *S. aureus* ATCC6538, and MRSA-Z were cultured in LB medium to their exponential phase (OD600 = 1.0), followed by dilution to 5×10^6 CFU/mL in PRMI 1640 medium containing 2% glucose. For biofilm inhibition assays, aliquots of bacterial solution (100 μL) and the test sample were mixed in a 96-well plate, followed by incubation at 37 °C for 24 h. For biofilm eradication assays, 100 μL of bacterial solution was first added to the wells of a 96-well plate, followed by incubation at 37 °C for 24 h. Then, the medium was discarded, and 100 μL of the test sample was added, followed by treatment for another 24 h. After the final incubation, all liquid was removed, and the wells were stained

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

Table 1. Physicochemical Properties and Antimicrobial Activities of TC-LAR-18 and Its Analogues[a]

peptide or controls	sequence	length	net charge	hydrophobic moment (μH)	MIC (μg/mL)			
					A. baumannii	*P. aeruginosa*	*E. coli*	*S. aureus*
TC-33	LLRRGGEKLAEKFEKIGQKIKNFFRKLLPETES	48	+4	0.542	75	37.5	150	75
TC-LAE-18	LAEKFEKIGQKIKNFFRK	30	+4	0.706	37.5	37.5	75	37.5
TC-LAR-18	LARKFRKIGQKIKNFFRK	18	+8	0.737	2.34	9.38	4.69	1.17
Colistin	-	-	-	-	0.59	2.34	0.59	
Vancomycin	-	-	-	-	-	-	-	1.17

[a]Four bacterial strains, including *A. baumannii* ATCC19606, *P. aeruginosa* ATCC27853, *E. coli* ATCC8739, and *S. aureus* ATCC6538, were used in the antibacterial assays. MICs represent mean values of three independent experiments. "-", not shown or determined.

with 150 μL of 0.1% crystal violet for 15 min. The dye was then removed, and the wells were washed with saline. Finally, 95% ethyl alcohol (200 μL) was used to resolubilize the stain, and the absorbance was measured at 600 nm using a microplate reader (Epoch, Bio Tek, USA).

2.7. Bacterial Killing Kinetics. Briefly, three strains of bacteria, including *P. aeruginosa* ATCC27853, *A. baumannii* ATCC19606, and *S. aureus* ATCC6538, were first cultured to their exponential phase (OD600 = 1.0), followed by dilution to 1×10^5 CFU/mL with the same medium and incubated with peptide or control [5 × minimal inhibitory concentration (MIC)]. At different time points (0, 1, 10, 30, 60, and 180 min), two microliter aliquots were taken out and diluted to an appropriate multiple with saline. The dilutions (50 μL) were then plated on LB agar plates and cultured overnight at 37 °C. The bacterial colonies were then counted and expressed as lg CFU/mL. Each test was conducted in triplicate.

2.8. Drug Resistance Assay. Liquid LB medium was used for passaging the bacteria every 24 h, following our previous research.[28,29] Throughout this process, the bacteria were cultured on LB medium with increasing concentrations of AMPs: 25% MIC for generations 1−10, 50% MIC for generations 11−20, 75% MIC for generations 21−30, 100% MIC for generations 31−40, 150% MIC for generations 41−50, and 200% MIC for generations 51−60. After 60 consecutive passages, the bacteria were taken from the culture at five-generation intervals, and the MIC values of the samples against the tested bacteria were determined.

2.9. Bacterial Membrane Permeabilization Assay. The effects of TC-LAR-18 on membrane permeabilization were measured by a fluorescence assay according to our previous report.[28] Briefly, logarithmic-phase bacteria cultured in LB broth were harvested by centrifuging and resuspended to 2×10^8 CFU/mL after washing three times with sterile normal saline. Thereafter, 100 μL of bacterial suspension was added to a black 96-well plate, and 10 μL of 50 μg/mL propidium iodide (PI) was also added to the wells. Subsequently, 100 μL of peptide (final concentrations of 1, 5, 10 × MIC) was added to the wells, followed by detecting the fluorescence changes at an excitation wavelength of 535 nm and an emission wavelength of 615 nm. Colistin (or vancomycin) in a final concentration of 5 × MIC was used as the positive control, and saline was used as the negative control.

2.10. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) Observation of the Morphological Changes of Bacteria. Logarithmic-phase bacteria (*P. aeruginosa* ATCC27853 and *S. aureus* ATCC6538) were resuspended to 2×10^9 CFU/mL after three times of washing with sterile normal saline. The resulting suspension was then incubated with the test samples in a concentration of 10 × MIC for 2 h at 37 °C. Subsequently, the cell pellets were obtained by centrifugation at 2000*g* for 5 min and fixed overnight at 4 °C using 2.5% glutaraldehyde. For SEM observations, the samples were dehydrated using a graded series of ethanol and tert-butanol, dried using liquid CO_2, sputtered with gold−palladium, and observed using SEM (Regulus 8220, Hitachi, Japan). For TEM observations, the samples were dehydrated with a graded series of ethanol, embedded with epoxy resin, sectioned with an ultramicrotome, stained with uranyl acetate and lead citrate, and finally observed using TEM (JEM-1400Flash, JEOL, Japan).

2.11. Acute Toxicity Analysis. Ten female C57BL/6 mice (6−8 weeks old) were intravenously injected with a single dose of TC-LAR-18 or controls (colistin and vancomycin) at 10 mg/kg. Saline was used as a negative control. Then, their behaviors were observed at different time points (10 min−96 h), as described in our previous studies.[28,29]

2.12. Mouse Skin Wound Infections. The therapeutic effects of TC-LAR-18 were evaluated using a mouse skin wound infection model following our previous study.[28] Briefly, healthy female BALB/c mice were first randomly divided into five groups with eight replicates per group (n = 8). After anesthetization, the dorsal skin of mice was shaved, disinfected with 75% ethanol, and cut with scissors to produce a round incision with 6 mm in diameter. Four hours after inoculating the skin wounds with 20 μL of *S. aureus* (6538) and *A. baumannii* (19606) at 1×10^8 CFU/mL, a 20 μL dose of TC-LAR-18 was then applied (0.1 to 2 mg/mL). Saline, colistin, and vancomycin (0.5 mg/mL) were used as controls. After 2 h, the incision area of the mice skin was excised, homogenized, and serially diluted in sterile saline. The dilutions were plated on LB agar plates and incubated overnight at 37 °C to quantify the number of bacterial colonies.

2.13. Statistical Analysis. Differences in mean values among different groups were determined and expressed as the mean ± standard deviation (SD). The Kolmogorov−Smirnov test was first used for normal distribution analysis, thereafter by a one-way analysis of variance (ANOVA) with post hoc Dunnett adjustment for p values. Data were analyzed using Prism v9.0 (GraphPad Software), and differences were considered significant at $p < 0.05$.

3. RESULTS

3.1. Design of TC-LAR-18 and Functional Screening. Cationicity is crucial for the direct antibacterial efficacy of AMPs, as it facilitates initial electrostatic interactions with negatively charged bacterial membrane components.[13] A strong correlation exists between cationicity and antimicrobial

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

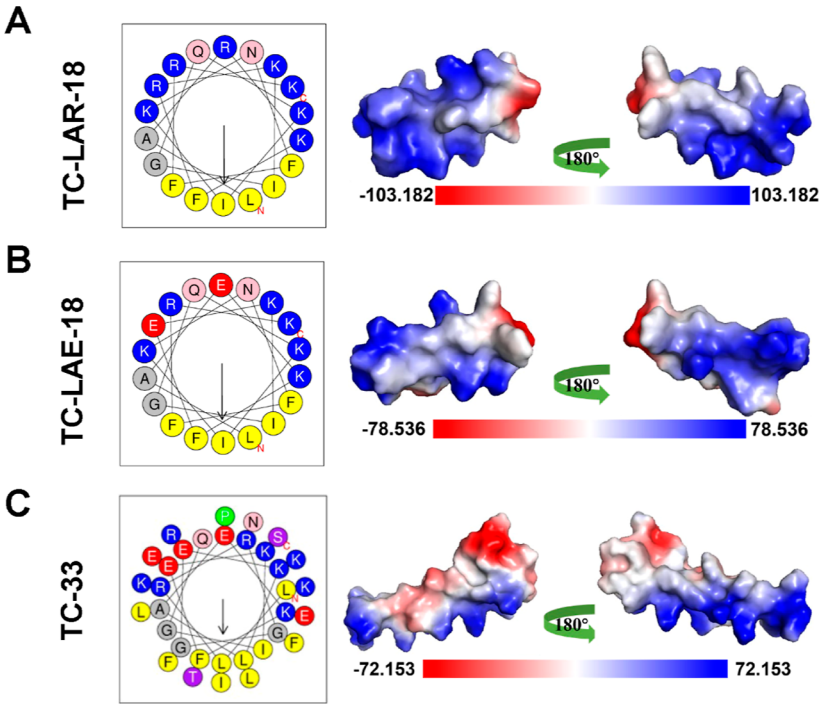


Figure 1. Helical wheel projections and surface electrostatic potentials of TC-LAR-18 and its analogues. Yellow indicates hydrophobic residues, blue represents positively charged hydrophilic residues, red represents glutamic acid, and gray denotes alanine and glycine. Surface electrostatic potentials were determined by PyMOL. Blue represents positive charge and red represents negative charge.

activity.[11] TC-33 contains 5 glutamates, which reduce its cationicity and likely contribute to its poor antimicrobial activity.[12,17] In addition, AMPs with short primary sequences show advantages including reduced cost of peptide synthesis, good permeability, high bioavailability, and low immunogenicity. We thus initially designed a smaller peptide, termed TC-LAE-18, by removing eight residues from the N-terminus and seven from the C-terminus of TC-33 (Table 1). TC-LAE-18, with two Glu residues at positions 3 and 6, with a net charge of +4 at pH 7. To enhance the cationicity of the peptide, we substituted the Glu residues at positions 3 and 6 in TC-LAE-18 with cationic residues. Compared to Lys, Arg substitution can yield enhanced antibacterial activity and may minimize the required length to achieve functional AMPs;[29,31,32] we thus chose Arg residue to substitute the Glu residue in TC-LAE-18, and creating TC-LAR-18 (Table 1). TC-LAR-18 exhibited high cationicity, exhibiting a net charge of +8 at physiological pH. In addition, TC-LAR-18 also showed a higher hydrophobic moment (μH) than TC-LAE-18 (Table 1). PyMOL was used to visualize the surface electrostatic potentials of TC-LAR-18, TC-LAE-18, and TC-33 (Figure 1). The amino acid substitution in TC-LAR-18 led to a significant increase in surface electrostatic potential compared with TC-LAE-18 and TC-33. Given the high charge, hydrophobic moment, and surface electrostatic potential of TC-LAR-18, we hypothesized that its antimicrobial activity would be further enhanced. To test this, we synthesized TC-LAR-18 and TC-LAE-18 with a C-terminal amide and evaluated their antimicrobial activity against four standard bacterial strains, including *A. baumannii*, *P. aeruginosa*, *E. coli*, and *S. aureus*. The MIC value of TC-33 against the tested bacteria[26] was also showed. As shown in Table 1, TC-LAE-18 exhibited antimicrobial activity similar to that of TC-33. However, TC-LAR-18 displayed significantly improved antimicrobial activity, with MICs of 1.17 to 9.38 μg/mL, representing a 4−64-fold and 4−128-fold increase in activity relative to TC-LAE-18 and TC-33, respectively.

Strong antimicrobial activity often correlates with cytotoxicity and hemolysis,[33−35] which are critical factors in therapeutic drug selection for in vivo studies.[36−38] Consequently, we evaluated the hemolytic and cytotoxic effects of TC-LAR-18 and TC-LAE-18. Despite the considerable differences in their antimicrobial activity (Table 1), neither peptide exhibited hemolytic (hemolysis at the highest concentration of 100 μg/mL was 0.73 and 1.61% for TC-LAR-18 and TC-LAE-18, respectively, Figure S1A,B) nor cytotoxic activity against human HaCaT keratinocytes (cell viability at the highest concentration of 100 μg/mL was 103.95 and 102.73% for TC-LAR-18 and TC-LAE-18, respectively, Figure S1C,D).

3.2. TC-LAR-18 Is Effective at Killing Planktonic and Biofilm-Associated Bacteria. To further study the antibacterial efficacy of TC-LAR-18, we tested its activity against 26 clinically isolated bacterial strains, including three strains of *A. baumannii*, four strains of *P. aeruginosa*, three strains of *E. coli*, seven strains of *S. aureus*, and nine strains of MRSA, which have shown varying degrees of drug resistance in our previous studies.[28,29] As illustrated in Table S1, TC-LAR-18 exhibited potent and broad-spectrum antibacterial activity against these drug-resistant clinical isolates, with MICs of 2.34 to 4.69, 4.69, and 2.34 to 4.69 μg/mL against *A. baumannii*, *P. aeruginosa*, *E. coli*, and *S. aureus*, respectively.

The role of bacterial biofilms in infectious diseases has garnered substantial attention.[39,40] Biofilm-forming bacteria are implicated in approximately 65−80% of infectious diseases.[41] Bacteria within biofilms can evade the immune system, making infections more challenging to treat. Furthermore, biofilms can survive on the surface of medical devices and implants, increasing the risk of infection when undergoing surgical or

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

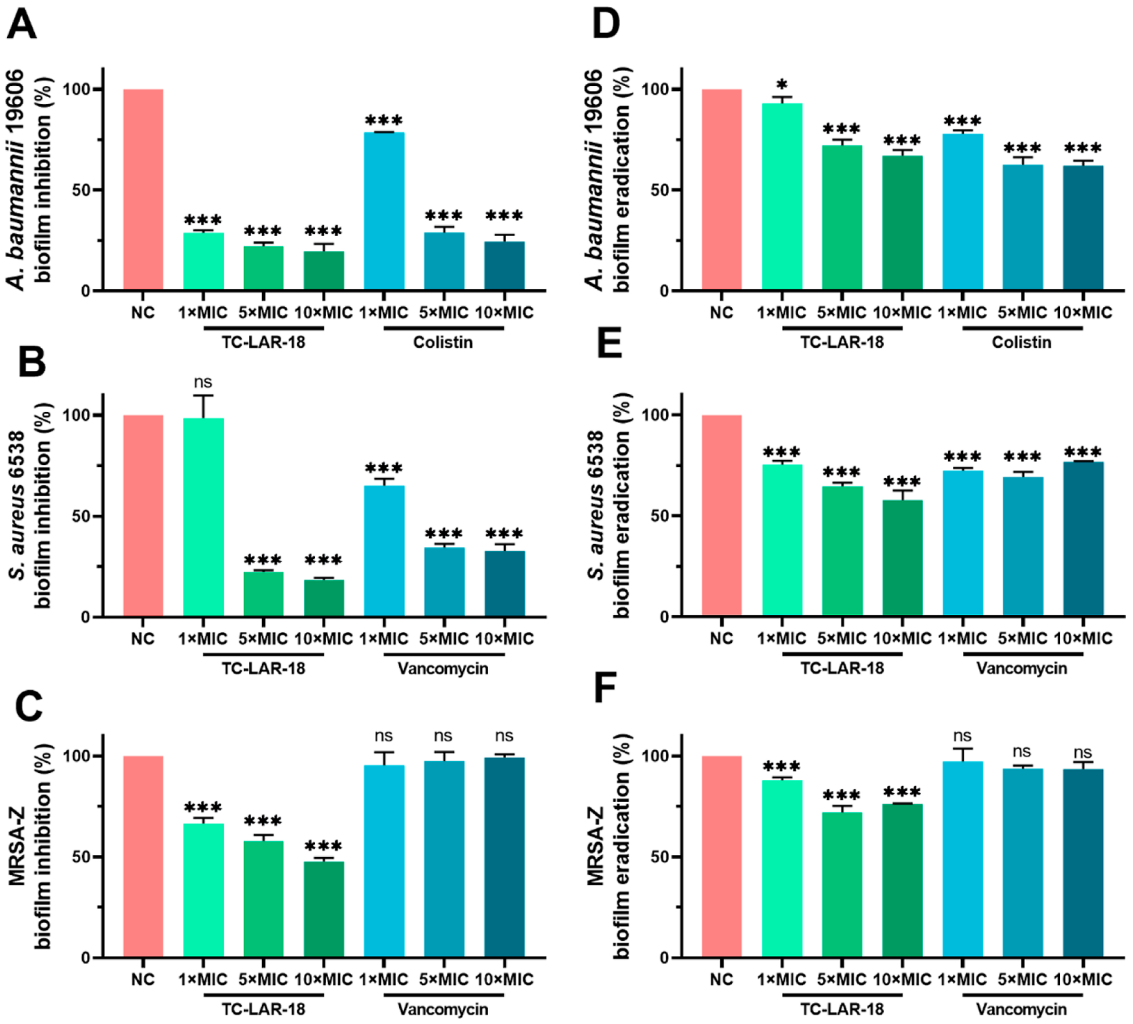


Figure 2. Biofilm inhibition and eradication. Biofilm inhibition and eradication of *A. baumannii* (19606) (A and D), *S. aureus* (6538) (B and E), and MRSA-Z (C and F). Negative control (NC) was saline. Data represent the mean ± SD of three individual experiments. One-way ANOVA with Dunnett post hoc test compared with NC. $*p < 0.05$, $***p < 0.001$, ns, no significant difference.

other medical procedures.[42−44] We evaluated the effects of TC-LAR-18 on biofilms formed by *A. baumannii*, *S. aureus*, and MRSA-Z. Notably, TC-LAR-18 significantly inhibited *A. baumannii* biofilm formation, showing greater activity than colistin (Figure 2A). Similarly, TC-LAR-18 markedly and dose-dependently inhibited the formation of *S. aureus* (Figure 2B) and MRSA-Z (Figure 2C) biofilm formation. Vancomycin, used as a positive control, was effective in inhibiting biofilm formation by *S. aureus* but not by MRSA-Z, likely due to its weaker antimicrobial activity against MRSA-Z (Table S1). An ideal antibiofilm agent should also disrupt preformed biofilms. As illustrated in Figure 2D−F, TC-LAR-18 eradicated the preformed *A. baumannii* (Figure 2D), *S. aureus* (Figure 2E), and MRSA-Z (Figure 2F) biofilms, although its activity was slightly less potent than its biofilm-eradicating activity. These findings suggest that TC-LAR-18 holds considerable potential in preventing bacterial infections due to its antimicrobial and antibiofilm properties.

3.3. TC-LAR-18 Shows Rapid Bactericidal Effects and Is Less Likely to Induce Resistance. To evaluate the bactericidal capacity of TC-LAR-18, bactericidal kinetic studies were performed using *P. aeruginosa*, *A. baumannii*, and *S. aureus*. TC-LAR-18 demonstrated time-dependent bactericidal activity, completely eradicating *P. aeruginosa* within 30 min (Figure 3A) and *A. baumannii* (Figure 3B) within 180 min at 5 × MIC. Colistin exhibited comparable activity against *P. aeruginosa* and *A. baumannii*. A TC-LAR-18 eradicated 99% of *S. aureus* cells within 180 min at 5 × MIC (Figure 3C), while vancomycin was less effective, likely due to its mechanism of inhibiting peptidoglycan synthesis without immediate bacterial killing.

Drug-resistant bacterial infections pose a significant threat to public health.[45] To evaluate the potential for drug resistance development to TC-LAR-18, we subjected the bacteria to serial passaging (up to 60 cycles) under exposure to varying concentrations of the peptide or controls. As illustrated in Figure 3D, *P. aeruginosa* exposed to TC-LAR-18 showed no propensity to develop resistant isolates, while colistin treatment led to a twofold increased MIC values after 30 passages. Unexpectedly, exposure of *S. aureus* to TC-LAR-18 resulted in a twofold increase in MIC values (Figure 3E), whereas vancomycin incubation resulted in a fourfold increased MIC.

3.4. TC-LAR-18 Induces Bacterial Membrane Permeabilization and Rupture. We next explored the effect of TC-LAR-18 on bacterial membrane permeabilization using a fluorescence assay. PI, a small fluorescent molecule, fluoresces more intensely upon interacting with DNA or RNA yet is unable to cross the plasma membrane of intact cells. As shown

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

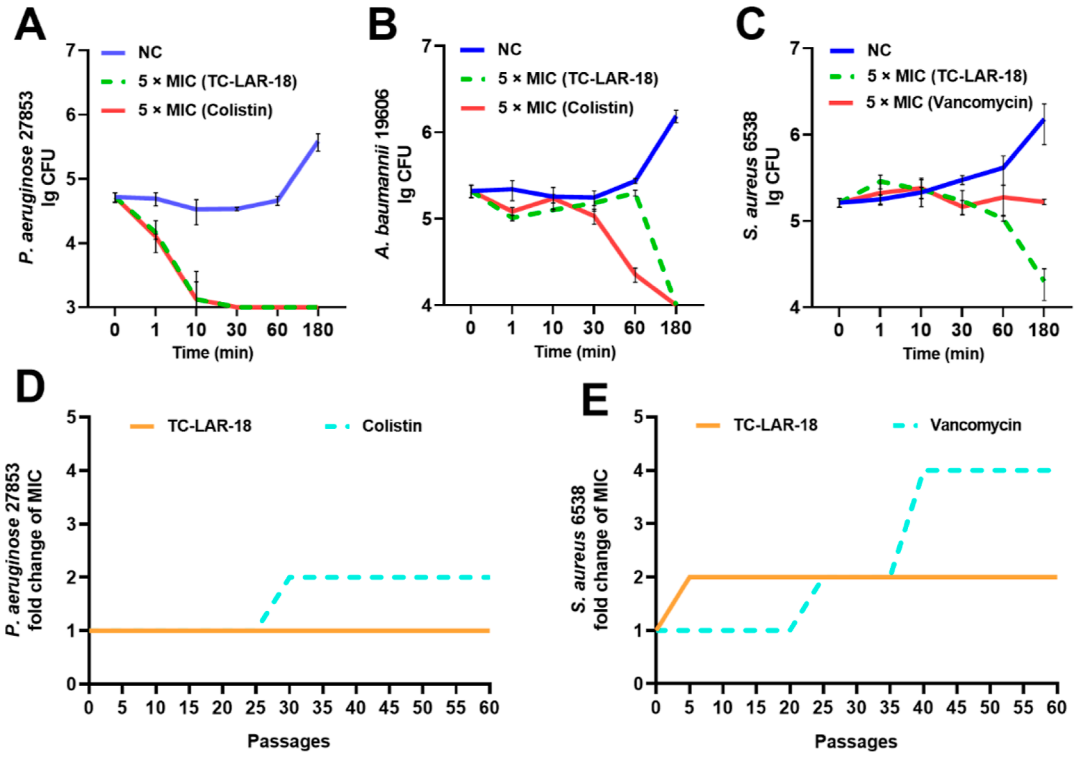


Figure 3. Bactericidal capacity of TC-LAR-18 in vitro and drug resistance. Bactericidal kinetics of TC-LAR-18 against *P. aeruginosa* (27853) (A), *A. baumannii* (19606) (B), and *S. aureus* (6538) (C). CFU: colony forming unit; NC: saline. Resistance development of *P. aeruginosa* (27853) (D) and *S. aureus* (6538) (E), following long-term exposure (60 generations) to the peptide, colistin, or vancomycin.

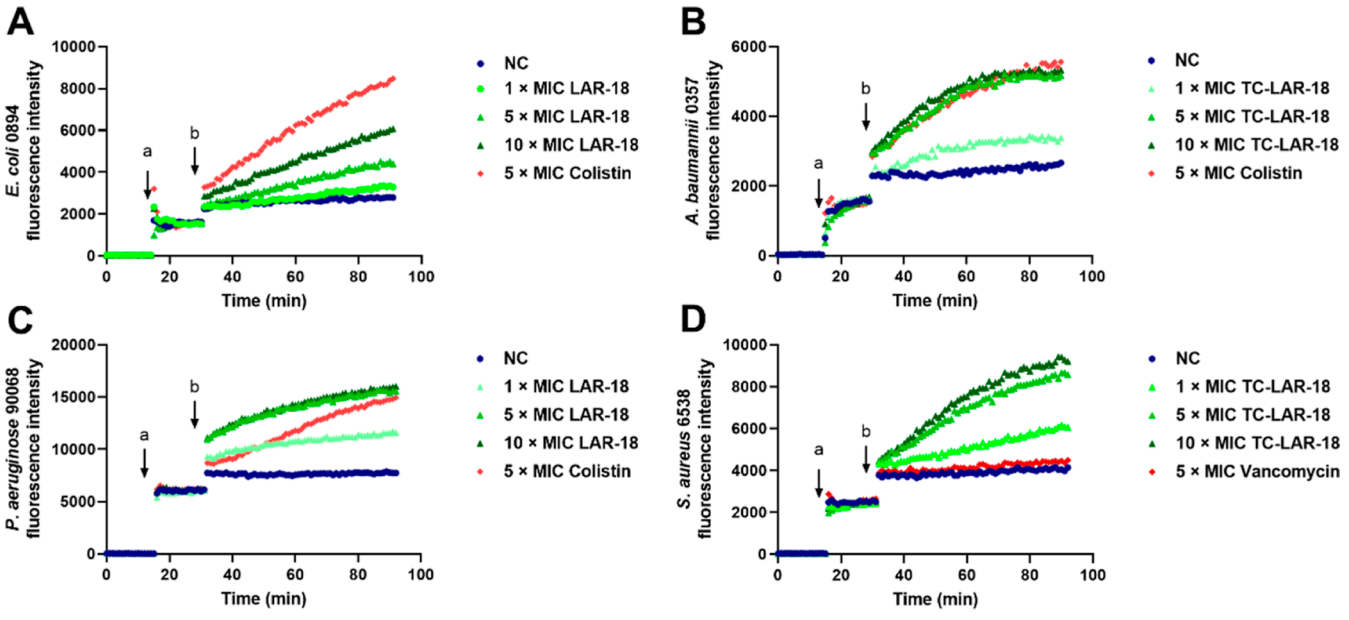


Figure 4. TC-LAR-18 induces bacterial plasma membrane permeabilization. *E. coli* (0894) (A), *A. baumannii* (0357) (B), *P. aeruginosa* (90068) (C), and *S. aureus* (6538) (D) were incubated with PI for 15 min, followed by the addition of test samples. Fluorescence intensities of PI were monitored over a 90 min period. Arrows indicate points where addition PI (a) and samples (b) were introduced. Data are representative measurements of three independent replicates.

in Figure 4A–D, PI fluorescence intensity increased immediately following the addition of TC-LAR-18, indicating rapid alteration in bacterial membrane permeability, which was positively correlated with the duration of exposure. Colistin exhibited comparable activity in inducing membrane permeabilization. In contrast, vancomycin did not significantly alter the membrane permeability, as evidenced by minimal changes in PI fluorescence intensity (Figure 4D).

We further investigated changes in the morphology of *P. aeruginosa* and *S. aureus* cells after TC-LAR-18 exposure using SEM and TEM. Normal *P. aeruginosa* treated with saline (vehicle) revealed rod-shaped with an intact and smooth surface (Figure 5A). However, TC-LAR-18-treated bacteria

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

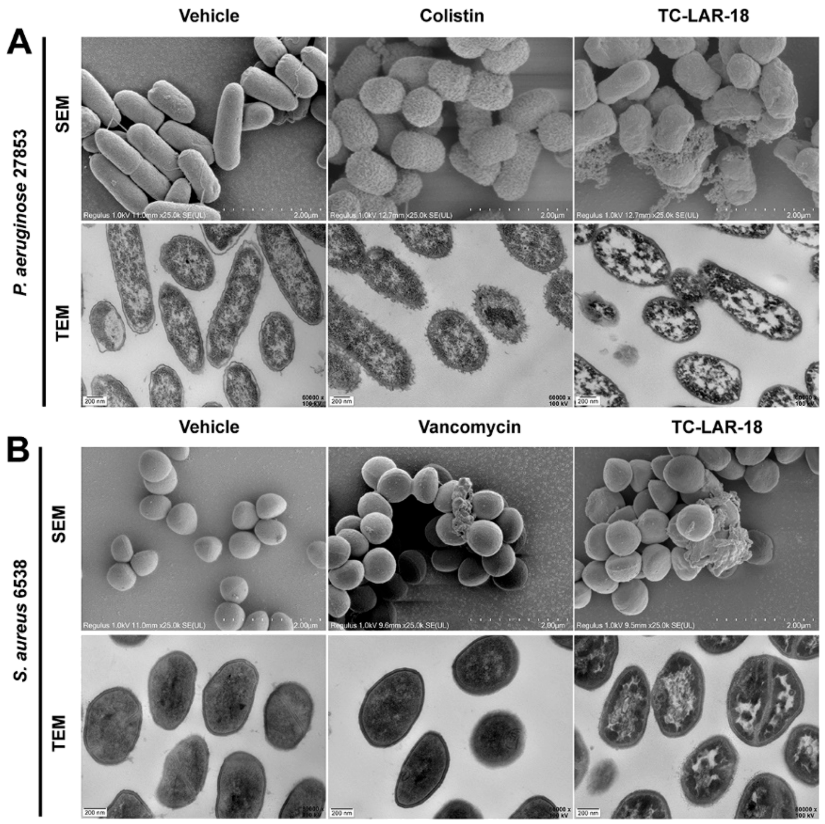


Figure 5. TC-LAR-18 induces bacterial membrane rupture. SEM and TEM images showing changes in *P. aeruginosa* (27853) (A) and *S. aureus* (6538) (B) morphology, following treatment with TC-LAR-18 or controls (5 × MIC). Saline treatment was used as a control. Images are representative of at least three individual replicates.

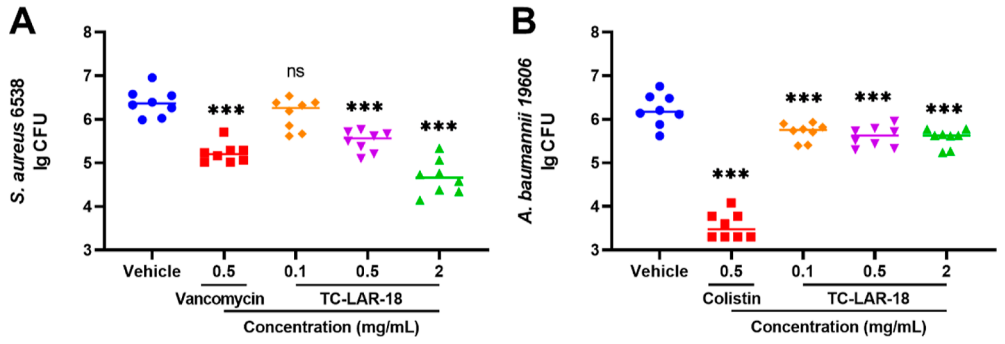


Figure 6. TC-LAR-18 exhibits protective effects against skin infection in mice. Female BALB/c mice were infected through skin wounds with either *S. aureus* (6538) (n = 8) (A) or *A. baumannii* (19606) (n = 8) (B) through skin wounds. At 4 h postinfection, 20 μL of TC-LAR-18 (0.1−2 mg/mL) or controls [saline, vancomycin, or colistin (0.5 mg/mL)] was then applied. Bacterial colonization levels were measured 2 h after sample application. One-way ANOVA with Dunnett post hoc test relative to vehicle group. ***p < 0.001, ns, no significant difference.

exhibited marked structural changes, including cell wall disruption and content leakage. TEM images further revealed cell membrane lysis with blurred borders accompanied by flocculent material. Colistin treatment led to significant cell wall irregularities and protrusions but without cellular leakage. Notably, TC-LAR-18 incubation of *S. aureus* resulted in visible structural changes, including cell wall rupture, leakage of bacterial inclusion bodies, blurring of cell membrane boundaries, and cytoplasmic clearing. Vancomycin treatment caused slight cell lysis and membrane blurring in *S. aureus* but no cytoplasmic clearing (Figure 5B). These findings indicate that TC-LAR-18 operates via a mechanism distinct from that of traditional antibiotics.

3.5. TC-LAR-18 Protects Mice against Skin Infection.

Toxicity and poor bioavailability are major barriers to the therapeutic application of AMPs.[46] Therefore, we performed an acute toxicity assessment of TC-LAR-18 before starting the in vivo experiments. As shown in Figure S2, administration of TC-LAR-18 and vancomycin (10 mg/kg) did not cause any observable signs of toxicity within a 96 h time frame. However, colistin administration at the same dose caused mild signs of toxicity in mice, such as ruffled fur and poor motility at 10 min, although these symptoms later resolved.

The skin serves as a barrier against mechanical damage, chemical, and exogenous substances.[47] Treating skin wounds topically presents specific challenges, such as maintaining a moist environment, managing localized pain, and addressing

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

the presence of inflammatory cells and molecules within the wound tissue.[48] These conditions are ideal for testing the therapeutic efficacy of AMPs. To assess the therapeutic efficacy of TC-LAR-18, we established a murine model of skin wound infection induced by *S. aureus* and *A. baumannii*. As shown in Figure 6A, TC-LAR-18 reduced the *S. aureus* load in a dose-dependent manner, achieving reductions of 88.6 and 97.8% in skin colonization at doses of 0.5 and 2 mg/mL, respectively. Vancomycin, used as a positive control, showed a 90.3% reduction in the colony counts at a dose of 0.5 mg/mL. A similar dose-dependent decrease in *A. baumannii* load was observed following TC-LAR-18 treatment (Figure 6B). Colistin, a first-line therapeutic drug, was more effective at reducing the bacterial load. These results indicate that topical application of TC-LAR-18 has significant therapeutic potential for treating skin infections.

4. DISCUSSION

AMPs are considered promising alternatives to traditional antibiotics for combating bacterial infections, with several already undergoing clinical trials.[49] Structure−activity relationship studies have elucidated the mechanisms by which AMP functions, primarily involving their binding to and penetration of bacterial cell membranes, leading to membrane lysis and cell death.[12,50] Initial bacterial membrane binding is predominantly driven by electrostatic interactions, while membrane permeation is facilitated by the hydrophobic characteristics of AMPs. The number of cationic residues and the overall net charge are crucial for antimicrobial potential because they enhance initial electrostatic interactions with negatively charged bacterial membrane components.[13] However, evidence suggests that an optimal range of positive charges (+4 to +8) exists for α-helical cationic AMPs, and deviation from this range can markedly reduce antimicrobial efficacy and increase hemolytic activity.[51] Most native AMPs possess a net charge within this optimal range, which likely maximizes their effectiveness.[5,21] In the present study, we first designed TC-LAE-18 by truncating the peptide to include two Glu residues at positions 3 and 6, resulting in a net charge of +4 at pH 7 (Table 1). Due to the weak antimicrobial activity of TC-LAE-18, we aimed to enhance its activity by increasing its net positive charge by substituting the two Glu residues with Arg, creating the highly cationic peptide TC-LAR-18, with a net charge of +8 at physiological pH (Table 1). TC-LAR-18 exhibited greater activity than TC-LAE-18 but showed no signs of cytotoxicity and hemolysis (Figure S1), likely because its net positive charge remained within the optimal range.[51] Our finding, along with previous observations, suggests that designing novel AMPs with a net charge of +8 is an effective strategy for maintaining antimicrobial activity while minimizing hemolytic and cytotoxic activity.

TC-14, another peptide analogue derived from TC-33, contains 14 amino acids and possesses a net positive charge of +5, which may serve as the active region of TC-33.[27] Although TC-LAR-18 exhibited similar antimicrobial activity against Gram-negative bacteria as TC-14, it exhibited fourfold increased potency against *S. aureus*, suggesting that alterations in peptide length and number of cationic residues can impact the antimicrobial activity and selectivity of AMPs. Using the parent AMP cathelicidin-BF, we designed a series of peptide derivatives with varying selectivity and activity, including LZ1, which is effective against *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *S. aureus*, all these bacteria play roles in the development of acne vulgaris,[52] ZY13, which exhibits strong antimicrobial activity against *Candida albicans*,[53] and ZY4, a cyclic peptide with an intramolecular disulfide bridge, which shows potent antimicrobial activity against *P. aeruginosa* and *A. baumannii*.[17] While AMPs show varied activity or selectivity, the precise structural determinants responsible for their selectivity remain to be elucidated. The differences in the bacterial cell membrane composition and interactions between AMPs and bacterial cell membrane components may significantly contribute to the selectivity of AMPs.

Most antibiotics target microbes through a single primary mechanism, which partly explains the high tendency for resistance development.[54] Generally, modifying their membrane composition is difficult and costly for most microorganisms, although recent studies have indicated that bacteria can induce resistance to AMPs.[55−57] In our study, we found that TC-LAR-18 killed bacteria by permeabilizing and disrupting the bacterial membrane (Figures 4 and 5), which may explain the low propensity of TC-LAR-18 to induce resistance (Figure 3D,E). Furthermore, TC-LAR-18 killed bacteria rapidly within 30−180 min (Figure 3A−C), resulting in fewer bacterial generations for resistance to evolve. Membrane integrity is essential for the survival of bacteria, irrespective of their metabolic stage. As TC-LAR-18 targeted bacterial membrane, it also showed high activity against biofilms. Recent studies have identified several Trp/Arg-containing AMPs that can kill biofilms.[16,17,58,59] Thus, antimicrobial agents with both antimicrobial and antibiofilm activities, such as TC-LAR-18, represent promising candidates for developing new antimicrobials. This study provides insight into the importance of positively charged residues for antibacterial activity and introduces a potential drug candidate for combating multidrug-resistant bacterial pathogens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06857.

Hemolytic and cytotoxic activity of TC-LAR-18 and TC-LAE-18; TC-LAR-18 *in vivo* toxicity; and antimicrobial activities of TC-LAR-18 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Wenlin Chen − *Third Department of Breast Surgery, Peking University Cancer Hospital Yunnan, Third Affiliated Hospital of Kunming Medical University (Yunnan Cancer Hospital), Kunming 650118 Yunnan, China*; Email: chenwenlin1@hotmail.com

Tianyu Zhang − *Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming 650031 Yunnan, China*; Email: zty20231989@163.com

Zhiye Zhang − *Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming 650031 Yunnan, China*; orcid.org/0000-0002-1661-2277; Email: zhangzhiye5225@163.com

Authors

Lin Luo − *Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming*

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288

650031 Yunnan, China; Third Department of Breast Surgery, Peking University Cancer Hospital Yunnan, Third Affiliated Hospital of Kunming Medical University (Yunnan Cancer Hospital), Kunming 650118 Yunnan, China

Ying Cai − *Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming 650031 Yunnan, China*

Yunhan Su − *School of Basic Medical Sciences, Kunming Medical University, Kunming 650500 Yunnan, China*

Chenxi Li − *Third Department of Breast Surgery, Peking University Cancer Hospital Yunnan, Third Affiliated Hospital of Kunming Medical University (Yunnan Cancer Hospital), Kunming 650118 Yunnan, China*

Gengzhou Tian − *Department of Breast Surgery, First Affiliated Hospital of Kunming Medical University, Kunming 650223 Yunnan, China*

Xingyu Wang − *Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming 650031 Yunnan, China; School of Life Sciences, Yunnan University, Kunming 650500, China*

Zhongxiang Wu − *Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming 650031 Yunnan, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c06857

Author Contributions

[#]L.L., Y.C., and Y.S. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the funding support from the National Natural Science Foundation of China (32070443), the Yunnan Province Grants (202302AA310015 and 202301AU070045), the Fundamental Research Funds for the Central Universities (3332023081), the Middle-aged and Young Academic and Technical Leader Reserve Talent Program of Yunnan Province Science and Technology Department (202205AC160008), and the Yunnan Province's Xing Dian Talent Support Program-Specialization in Medical and Health Talents.

■ REFERENCES

(1) Kędziora, A.; Wernecki, M.; Korzekwa, K.; Speruda, M.; Gerasymchuk, Y.; Łukowiak, A.; Bugla-Płoskońska, G. Consequences of long-term bacteria's exposure to silver nanoformulations with different physicochemical properties. *Int. J. Nanomed.* **2020**, *15*, 199−213.

(2) Khardori, N.; Stevaux, C.; Ripley, K. Antibiotics: From the beginning to the future: part 2. *Indian J. Pediatr.* **2020**, *87* (1), 43−47.

(3) Thiemann, S.; Smit, N.; Strowig, T. Antibiotic resistance: Problems and new opportunities. In *How to Overcome the Antibiotic Crisis Facts, Challenges, Technologies and Future Perspectives*; Springer, 2016; Vol. *398*, pp 1−236.

(4) Pendleton, J. N.; Gorman, S. P.; Gilmore, B. F. Clinical relevance of the ESKAPE pathogens. *Expert Rev. Anti-Infect. Ther.* **2013**, *11* (3), 297−308.

(5) Wang, G.; Li, X.; Wang, Z. APD3: The antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res.* **2016**, *44* (D1), D1087−D1093.

(6) Lee, T. H.; Hofferek, V.; Separovic, F.; Reid, G. E.; Aguilar, M. I. The role of bacterial lipid diversity and membrane properties in modulating antimicrobial peptide activity and drug resistance. *Curr. Opin. Chem. Biol.* **2019**, *52*, 85−92.

(7) Jangra, M.; Kaur, M.; Nandanwar, H. *In-vitro* studies on a natural lantibiotic, paenibacillin: A new-generation antibacterial drug candidate to overcome multi-drug resistance. *Int. J. Antimicrob. Agents* **2019**, *53* (6), 838−843.

(8) Manniello, M. D.; Moretta, A.; Salvia, R.; Scieuzo, C.; Lucchetti, D.; Vogel, H.; Sgambato, A.; Falabella, P. Insect antimicrobial peptides: Potential weapons to counteract the antibiotic resistance. *Cell. Mol. Life Sci.* **2021**, *78* (9), 4259−4282.

(9) Patocka, J.; Nepovimova, E.; Klimova, B.; Wu, Q.; Kuca, K. Antimicrobial peptides: Amphibian host defense peptides. *Curr. Med. Chem.* **2019**, *26* (32), 5924−5946.

(10) Deo, S.; Turton, K. L.; Kainth, T.; Kumar, A.; Wieden, H. J. Strategies for improving antimicrobial peptide production. *Biotechnol. Adv.* **2022**, *59*, 107968.

(11) Han, Y.; Zhang, M.; Lai, R.; Zhang, Z. Chemical modifications to increase the therapeutic potential of antimicrobial peptides. *Peptides* **2021**, *146*, 170666.

(12) Mwangi, J.; Hao, X.; Lai, R.; Zhang, Z. Y. Antimicrobial peptides: New hope in the war against multidrug resistance. *Zool. Res.* **2019**, *40* (6), 488−505.

(13) Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415* (6870), 389−395.

(14) Yang, Y.; Huang, C.; Hui, L.; Song, Y.; Fu, Y.; Li, M.; Yang, H.; Wu, J.; Sun, J.; Xu, W.; et al. Cathelicidins target HSP60 to restrict CVB3 transmission via disrupting the exosome and reducing cardiomyocyte apoptosis. *J. Virol.* **2023**, *97* (3), No. e0143322.

(15) Sowers, A.; Wang, G.; Xing, M.; Li, B. Advances in antimicrobial peptide discovery via machine learning and delivery via nanotechnology. *Microorganisms* **2023**, *11* (5), 1129.

(16) de Breij, A.; Riool, M.; Cordfunke, R. A.; Malanovic, N.; de Boer, L.; Koning, R. I.; Ravensbergen, E.; Franken, M.; van der Heijde, T.; Boekema, B. K.; et al. The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms. *Sci. Transl. Med.* **2018**, *10* (423), No. eaan4044.

(17) Mwangi, J.; Yin, Y.; Wang, G.; Yang, M.; Li, Y.; Zhang, Z.; Lai, R. The antimicrobial peptide ZY4 combats multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infection. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (52), 26516−26522.

(18) Fuente-Núñez, C. d. l.; Silva, O. N.; Lu, T. K.; Franco, O. L. Antimicrobial peptides: Role in human disease and potential as immunotherapies. *Pharmacol. Ther.* **2017**, *178*, 132−140.

(19) Mookherjee, N.; Anderson, M. A.; Haagsman, H. P.; Davidson, D. J. Antimicrobial host defence peptides: Functions and clinical potential. *Nat. Rev. Drug Discovery* **2020**, *19* (5), 311−332.

(20) Koo, H. B.; Seo, J. Antimicrobial peptides under clinical investigation. *Pept. Sci.* **2019**, *111* (5), No. e24122.

(21) Shi, G.; Kang, X.; Dong, F.; Liu, Y.; Zhu, N.; Hu, Y.; Xu, H.; Lao, X.; Zheng, H. DRAMP 3.0: an enhanced comprehensive data repository of antimicrobial peptides. *Nucleic Acids Res.* **2022**, *50* (D1), D488−D496.

(22) Wang, G.; Narayana, J. L.; Mishra, B.; Zhang, Y.; Wang, F.; Wang, C.; Zarena, D.; Lushnikova, T.; Wang, X. Design of Antimicrobial Peptides: Progress Made with Human Cathelicidin LL-37. *Adv. Exp. Med. Biol.* **2019**, *1117*, 215−240.

(23) Wang, G.; Hanke, M. L.; Mishra, B.; Lushnikova, T.; Heim, C. E.; Chittezham Thomas, V.; Bayles, K. W.; Kielian, T. Transformation of human cathelicidin LL-37 into selective, stable, and potent antimicrobial compounds. *ACS Chem. Biol.* **2014**, *9* (9), 1997−2002.

(24) Wang, Y. P.; Hong, J.; Liu, X. H.; Yang, H. L.; Liu, R.; Wu, J.; Wang, A. L.; Lin, D. H.; Lai, R. Snake Cathelicidin from Bungarus fasciatus Is a Potent Peptide Antibiotics. *PLoS One* **2008**, *3* (9), No. e3217.

(25) Wang, Y.; Zhang, Z.; Chen, L.; Guang, H.; Li, Z.; Yang, H.; Li, J.; You, D.; Yu, H.; Lai, R. Cathelicidin-BF, a snake cathelicidin-derived antimicrobial peptide, could be an excellent therapeutic agent for acne vulgaris. *PLoS One* **2011**, *6* (7), No. e22120.

(26) Li, C.; Cai, Y.; Luo, L.; Tian, G.; Wang, X.; Yan, A.; Wang, L.; Wu, S.; Wu, Z.; Zhang, T.; et al. TC-14, a cathelicidin-derived

antimicrobial peptide with broad-spectrum antibacterial activity and high safety profile. *iScience* **2024**, *27* (7), 110404.
(27) Lamiable, A.; Thévenet, P.; Rey, J.; Vavrusa, M.; Derreumaux, P.; Tufféry, P. PEP-FOLD3: Faster de novo structure prediction for linear peptides in solution and in complex. *Nucleic Acids Res.* **2016**, *44* (W1), W449−W454.
(28) Cai, Y.; Wang, X.; Zhang, T.; Yan, A.; Luo, L.; Li, C.; Tian, G.; Wu, Z.; Wang, X.; Shen, D.; et al. Rational design of a potent antimicrobial peptide based on the active region of a *Gecko* cathelicidin. *ACS Infect. Dis.* **2024**, *10* (3), 951−960.
(29) Zhang, Z.; Chen, Y.; Gao, J.; Yang, M.; Zhang, D.; Wang, L.; Zhang, T.; Cao, Q.; Mwangi, J.; He, C.; et al. Orientational nanoconjugation with gold endows marked antimicrobial potential and drugability of ultrashort dipeptides. *Nano Lett.* **2023**, *23* (24), 11874−11883.
(30) Ye, Z.; Fu, L.; Li, S.; Chen, Z.; Ouyang, J.; Shang, X.; Liu, Y.; Gao, L.; Wang, Y. Synergistic collaboration between AMPs and non-direct antimicrobial cationic peptides. *Nat. Commun.* **2024**, *15* (1), 7319.
(31) Agrillo, B.; Porritiello, A.; Gratino, L.; Balestrieri, M.; Proroga, Y. T.; Mancusi, A.; Cozzi, L.; Vicenza, T.; Dardano, P.; Miranda, B.; et al. Antimicrobial activity, membrane interaction and structural features of short arginine-rich antimicrobial peptides. *Front. Microbiol.* **2023**, *14*, 1244325.
(32) Deslouches, B.; Hasek, M. L.; Craigo, J. K.; Steckbeck, J. D.; Montelaro, R. C. Comparative functional properties of engineered cationic antimicrobial peptides consisting exclusively of tryptophan and either lysine or arginine. *J. Med. Microbiol.* **2016**, *65* (6), 554−565.
(33) Powers, J. P.; Hancock, R. E. The relationship between peptide structure and antibacterial activity. *Peptides* **2003**, *24* (11), 1681−1691.
(34) Ding, B.; Guan, Q.; Walsh, J. P.; Boswell, J. S.; Winter, T. W.; Winter, E. S.; Boyd, S. S.; Li, C.; Savage, P. B. Correlation of the antibacterial activities of cationic peptide antibiotics and cationic steroid antibiotics. *J. Med. Chem.* **2002**, *45* (3), 663−669.
(35) Bobone, S.; Stella, L. Selectivity of Antimicrobial Peptides: A Complex Interplay of Multiple Equilibria. *Adv. Exp. Med. Biol.* **2019**, *1117*, 175−214.
(36) Costa, F.; Teixeira, C.; Gomes, P.; Martins, M. C. L. Clinical Application of AMPs. *Adv. Exp. Med. Biol.* **2019**, *1117*, 281−298.
(37) Henninot, A.; Collins, J. C.; Nuss, J. M. The Current State of Peptide Drug Discovery: Back to the Future? *J. Med. Chem.* **2018**, *61* (4), 1382−1414.
(38) Lau, J. L.; Dunn, M. K. Therapeutic peptides: Historical perspectives, current development trends, and future directions. *Bioorg. Med. Chem.* **2018**, *26* (10), 2700−2707.
(39) Shahrour, H.; Ferrer-Espada, R.; Dandache, I.; Barcena-Varela, S.; Sanchez-Gomez, S.; Chokr, A.; Martinez-de-Tejada, G. AMPs as Anti-biofilm Agents for Human Therapy and Prophylaxis. *Adv. Exp. Med. Biol.* **2019**, *1117*, 257−279.
(40) Fisher, R. A.; Gollan, B.; Helaine, S. Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* **2017**, *15* (8), 453−464.
(41) Lebeaux, D.; Chauhan, A.; Rendueles, O.; Beloin, C. From *in vitro* to *in vivo* models of bacterial biofilm-related infections. *Pathogens* **2013**, *2* (2), 288−356.
(42) Kannappan, A.; Srinivasan, R.; Nivetha, A.; Annapoorani, A.; Pandian, S. K.; Ravi, A. V. Anti-virulence potential of 2-hydroxy-4-methoxybenzaldehyde against methicillin-resistant *Staphylococcus aureus* and its clinical isolates. *Appl. Microbiol. Biotechnol.* **2019**, *103* (16), 6747−6758.
(43) Kannappan, A.; Durgadevi, R.; Srinivasan, R.; Lagoa, R. J. L.; Packiavathy, I.; Pandian, S. K.; Veera Ravi, A. 2-Hydroxy-4-methoxybenzaldehyde from hemidesmus indicus is antagonistic to *Staphylococcus* epidermidis biofilm formation. *Biofouling* **2020**, *36* (5), 549−563.
(44) Kalia, V. C.; Patel, S. K. S.; Lee, J. K. Bacterial biofilm inhibitors: An overview. *Ecotoxicol. Environ. Saf.* **2023**, *264*, 115389.
(45) Roope, L. S. J.; Smith, R. D.; Pouwels, K. B.; Buchanan, J.; Abel, L.; Eibich, P.; Butler, C. C.; Tan, P. S.; Walker, A. S.; Robotham, J. V.; et al. The challenge of antimicrobial resistance: What economics can contribute. *Science* **2019**, *364* (6435), No. eaau4679.
(46) Greco, I.; Molchanova, N.; Holmedal, E.; Jenssen, H.; Hummel, B. D.; Watts, J. L.; Håkansson, J.; Hansen, P. R.; Svenson, J. Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. *Sci. Rep.* **2020**, *10* (1), 13206.
(47) Smith, R.; Russo, J.; Fiegel, J.; Brogden, N. Antibiotic delivery strategies to treat skin infections when innate antimicrobial defense fails. *Antibiotics* **2020**, *9* (2), 56.
(48) Punjataewakupt, A.; Napavichayanun, S.; Aramwit, P. The downside of antimicrobial agents for wound healing. *Eur. J. Clin. Microbiol. Infect. Dis.* **2019**, *38* (1), 39−54.
(49) Lazzaro, B. P.; Zasloff, M.; Rolff, J. Antimicrobial peptides: Application informed by evolution. *Science* **2020**, *368* (6490), No. eaau5480.
(50) Zhang, Q. Y.; Yan, Z. B.; Meng, Y. M.; Hong, X. Y.; Shao, G.; Ma, J. J.; Cheng, X. R.; Liu, J.; Kang, J.; Fu, C. Y. Antimicrobial peptides: Mechanism of action, activity and clinical potential. *Mil. Med. Res.* **2021**, *8* (1), 48.
(51) Jiang, Z.; Vasil, A. I.; Hale, J.; Hancock, R. E.; Vasil, M. L.; Hodges, R. S. Effects of net charge and the number of positively charged residues on the biological activity of amphipathic α-helical cationic antimicrobial peptides. *Adv. Exp. Med. Biol.* **2009**, *611*, 561−562.
(52) Zhang, Z.; Mu, L.; Tang, J.; Duan, Z.; Wang, F.; Wei, L.; Rong, M.; Lai, R. A small peptide with therapeutic potential for inflammatory acne vulgaris. *PLoS One* **2013**, *8* (8), No. e72923.
(53) Jin, L.; Bai, X.; Luan, N.; Yao, H.; Zhang, Z.; Liu, W.; Chen, Y.; Yan, X.; Rong, M.; Lai, R.; et al. A designed tryptophan- and lysine/arginine-rich antimicrobial peptide with therapeutic potential for clinical antibiotic-resistant *Candida albicans* vaginitis. *J. Med. Chem.* **2016**, *59* (5), 1791−1799.
(54) Brazas, M. D.; Hancock, R. E. Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance. *Drug discovery today* **2005**, *10* (18), 1245−1252.
(55) Li, M.; Cha, D. J.; Lai, Y.; Villaruz, A. E.; Sturdevant, D. E.; Otto, M. The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Mol. Microbiol.* **2007**, *66* (5), 1136−1147.
(56) Abdi, M.; Mirkalantari, S.; Amirmozafari, N. Bacterial resistance to antimicrobial peptides. *J. Pept. Sci.* **2019**, *25* (11), No. e3210.
(57) Joo, H. S.; Otto, M. Mechanisms of resistance to antimicrobial peptides in *staphylococci*. *Biochim. Biophys. Acta* **2015**, *1848* (11), 3055−3061.
(58) Overhage, J.; Campisano, A.; Bains, M.; Torfs, E. C.; Rehm, B. H.; Hancock, R. E. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* **2008**, *76* (9), 4176−4182.
(59) Rajasekaran, G.; Kim, E. Y.; Shin, S. Y. LL-37-derived membrane-active FK-13 analogs possessing cell selectivity, anti-biofilm activity and synergy with chloramphenicol and anti-inflammatory activity. *Biochim. Biophys. Acta, Biomembr.* **2017**, *1859* (5), 722−733.

https://doi.org/10.1021/acsomega.4c06857
ACS Omega 2024, 9, 45279−45288