ACS Infectious Diseases

Article

Discovery of linear low-cationic peptides to target methicillin-resistant Staphylococcus aureus in vivo

Yuan Liu, Meirong Song, Shuangyang Ding, and Kui Zhu

ACS Infect. Dis., **Just Accepted Manuscript** • DOI: 10.1021/acsinfecdis.8b00230 • Publication Date (Web): 29 Oct 2018

Just Accepted

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Discovery of linear low-cationic peptides to target methicillin-resistant *Staphylococcus aureus in vivo*

Yuan Liu,[†] Meirong Song,[‡] Shuangyang Ding,[‡,§] Kui Zhu*[,†,‡,§]

[†]Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agricultural University, No.2 Yuanmingyuan West Road, Haidian, Beijing, China 100193

[‡]National Center for Veterinary Drug Safety Evaluation, College of Veterinary Medicine, China Agricultural University, No.2 Yuanmingyuan West Road, Haidian, Beijing, China 100193

[§]Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety and Beijing Laboratory for Food Quality and Safety, China Agricultural University, No.2 Yuanmingyuan West Road, Haidian, Beijing, China 100193

*Correspondence author: zhukcau@gmail.com (K.Z.).

The development and rapid spread of multi-drug resistant (MDR) bacteria causes a severe public crisis. New antibacterial compounds are urgently needed to treat bacterial infections. By circumventing the disadvantages of cationic peptides, here we engineered a short, linear, low cationic peptide bacaucin-1a, which exhibited remarkable antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Bacaucin-1a was efficient to prevent MRSA associated infections in both *in vitro* and *in vivo* models with unique mode of action. The discovery of low cationic antibiotic candidates will extend our antibiotic pipeline for fighting antibiotic resistant bacteria.

Keywords: antibacterial, antibiotic, MRSA, peptides

The global spread of MDR bacteria poses a severe threat to worsen public health.[1-3] To address this challenge, an efficient strategy is to develop novel antibiotics to directly combat pathogens with different modes of action.[4-9] Antimicrobial peptides and their analogs are potentially effective candidates against pathogens.[10-13] Such peptide-derivatives including lipopeptides, glycopeptides, lantibiotics, short peptides and other variants,[14,15] are produced by diverse organisms to compete with pathogenic microbes, to facilitate signal transduction, or to serve as physiological cues modulating population dynamics. Compared to these natural analogs, some of their derivatives play a crucial role in treating bacteria associated infections in the clinic. For example, glycopeptides such as vancomycin,[16] and lipopeptides such as polymyxins[17] are currently used as the last resort antibiotics against Gram-positive and Gram-negative bacteria, respectively. However, bacterial pathogens have evolved resistance rapidly due to the overuse and misuse of these antibiotics. For instance, bacteria can modify the negatively charged lipid A on the surface with a positively charged ethanolamine to resist cationic polymyxins.[18] Thus, there is a great need to develop new peptide-based antibiotic leads to restrict the emergence of antibiotic resistant bacterial pathogens.

Nowadays, synthetic short peptides with antibacterial activities have attracted more attentions. Such peptides are easily generated through the process of solid-phase peptide synthesis, and are readily accessible for further chemical modifications. However, despite several synthetic peptide leads have entered clinical trials,[19,20] three crucial obstacles facing to develop new generation of synthetic peptide-based antibiotics are cost-ineffective for chemical synthesis, poor protease stability and nonspecific toxicity.[21,22] Compared to the complicated natural structures, antibacterial short peptides can dramatically reduce the production cost. Several attempts have achieved promising results for treating bacterial infections in both *in vitro* and *in vivo* models such as MP196 (a synthetic hexapeptide, strongly enriched in tryptophan and arginine residues)[15], SNAPPs (structurally nanoengineered antimicrobial peptide polymers)[23] and CS-g-K_{16} (a cationic peptidopolysaccharide).[24] Such short antibacterial peptides and the derivatives always contain multiple positive charged amino acid residues that are ideal for binding to the negatively charged

bacterial surface.[25] Nevertheless, these peptides with intrinsic cationic-rich properties are also easily resulted in off-target toxicity under physiological conditions,[26,27] due to the nonspecific electrostatic interaction between the positively charged groups in the peptides and the abundant negatively charged biomacromolecules on cell surfaces. Therefore, we hypothesized that the short and linear peptide analogs with low cationicity would be more potential, especially in terms of stability and safety.

It is advantageous to look back into the history of previously known but undeveloped compounds for discovering new antibiotics. The family of cyclic noncationic lipopeptides from *Bacillus* species are great examples,[28] which includes surfactin, lichenysins, iturin and other classes. They all contain multiple negatively charged amino acid residues in lieu of positive ones in the chiral peptide sequences,[29] suggesting that they share different modes of action. Taken together, these examples further indicated that the potential of low cationic compounds can be used to combat MDR bacteria. Inspired by these positive evidence and our unflagging effort in developing surfactin-inspired antibiotics to combat MDR bacterial pathogens, we herein reported a short, linear, low-cationic heptapeptide antibiotic composed of all natural amino acid residues termed bacaucin-1a, which exhibited specific antibacterial activity against *Staphylococcus aureus*, especially for methicillin-resistant *Staphylococcus aureus* (MRSA) in both *in vitro* and *in vivo*.

Results and discussion

Screening linear low-cationic peptides. Bacaucin-1, H-ELLSRVD-OH,[30] was chosen as a model to achieve our goal in this study. We first used the *L*-alanine (Ala, A) screening method to investigate the role of each amino acid residue in antibacterial activity against *S. aureus* ATCC 29213 (**Figure 1A**). The replacement of Ala, the smallest chiral amino acid, will keep the basic structure of original peptide and indicate the function of side chains in amino acid residues. Interestingly, we found that the sole substitution of *L*-arginine (Arg, R) at position 5 totally lost antibacterial activity (**Figure S1**). To further elucidate the structure-activity relationship (SAR) of the side chains (R groups), we synthesized 39 *L*-type derivatives of bacaucin-1 based on their

polarities and electronic properties (**Table S1**). All these compounds were tested for antibacterial activity against *S. aureus* ATCC 29213. We found that the sole substitution of hydrophilic and negatively charged *L*-glutamic acid (Glu, E) by hydrophobic A at position 1 shows the best antibacterial activity with minimum inhibitory concentration (MIC) of 2 μg mL^{-1} (**Figure 1B**). Hence, this derivative of bacaucin-1 was named as bacaucin-1a, with negatively charged potential surface and measured zeta potential of -6.08 ± 0.19 mv (**Figure 1C**). Altogether, it suggest that bacaucin-1a is a low-cationic peptide with antibacterial activity against *S. aureus*. Additionally, compared to the all *L*-type one, the derivatives of bacaucin-1a with all *D*-type or partial *D*-type amino acid residues show a 4-fold to 32-fold reduced antibacterial activity (**Table S3**).

Guanidine group is crucial for antibacterial activity in bacaucin-1a. Compared to the other analogs of R groups at position 5 (**Figure 1B**), we found both the length of side chains and the positively charged guanidine group are crucial for antibacterial activity. To elaborate the function of guanidine group, we used 1,2-cyclohexanedione to block the guanidine group of arginine and get the derivatization of cyclohexanedione-bacaucin-1a conjugate (CBC). The purity of the conjugate product was 97.05% based on ultra-performance liquid chromatography-tandem mass (UPLC-MS/MS) analysis (**Figure 2A)**. As expected, CBC completely lost its antibacterial activity (**Figure 2B**), which confirmed the crucial role of the guanidine group in its antibacterial activity. Additionally, significant reduced activity (more than 64-fold) was observed when "C=NH" (arginine) was replaced by "C=O" (citrulline) (**Figure 1B**), suggesting that the imino in the guanidine group plays a crucial role in the antibacterial activity of bacaucin-1a.

Stability and safety assessment of bacaucin-1a. We next sought to evaluate the protease resistance and serum stability of bacaucin-1a, and found that bacaucin-1a is resistant to proteinase K, trypsin, pepsin, papain and 50% fetal bovine serum, superior to bacaucin-1 (**Table S4**). To further elaborate the mechanism of bacaucin-1a resistant to trypsin, we evaluated the resistance of bacaucin-1a and its substitutions to trypsin by UPLC-MS/MS method (**Figure 2C**). The substitutions of either *L*-serine (Ser, S) or *L*-valine (Val, V) dramatically increased the

percentage of hydrolyzed product under trypsin treatment (**Figure 2D**), suggesting that the neighboring serine and/or valine residues around arginine might contribute to the resistance. Furthermore, bacaucin-1a maintained antibacterial activity after heating at 80 °C for 1 h and exhibited high stability under physiological conditions with pH ranging from 6 to 8 (**Figure S2)**. Lastly, we evaluated the toxicity of bacaucin-1a on red blood cells and mammalian cells. No hemolysis and no cytotoxicity were observed at the concentration of bacaucin-1a above 50-fold of MIC value (**Figure S3)**. Taken together, these findings indicate that low cationic bacaucin-1a as a lead structure is promising for the development of new antibacterial agent.

Antibacterial spectrum of bacaucin-1a. Bacaucin-1a exhibited high specificity to *S. aureus*, MRSA and MDR-SA isolates, instead of other bacterial species (**Table S5**). To further confirm the antibacterial activity of bacaucin-1a, 100 clinical MRSA isolates from both human and animal origins were tested. Compared to daptomycin which exhibits the MIC values ranged from 0.25 μg mL^{-1} to 2 μg mL^{-1}, the MIC values of bacaucin-1a ranged from 2 μg mL^{-1} to 4 μg mL^{-1} against all tested isolates (**Figure 3A, Table S6**). We defined the coefficient of variance, which was the ratio of MIC_{max} to MIC_{min} of an antibiotic. The value of coefficient of bacaucin-1a was much lower than daptomycin, suggesting that the antibacterial activity of bacaucin-1a was more stable for diverse MRSA isolates (**Figure 3B**). To evaluate whether bacaucin-1a potentiates the activity of other antibiotics, five antibiotics from different classes were tested in the combination with bacaucin-1a. Bacaucin-1a shown synergy with ampicillin and rifampicin especially for rifampicin, but not for the other tested antibiotics (**Figure S4**). To assess whether this synergy was strain-specific, we determined six other rifampicin-resistant isolates, including four MRSA isolates, and one of each *Enterococcus faecalis* and *Escherichia coli*. Results showed that the synergy was species specific (**Figure S5**), which indicates that the potentiation of rifampicin in *S. aureus* might be associated with the mode of action of bacaucin-1a.

Mode of action of bacaucin-1a. To study the underlying mechanism, we first focused on the configuration of bacaucin-1a based on dynamic light scattering and nuclear magnetic resonance (NMR) analysis. No significant intensity changes was observed in the 26-hour continuous

measurement (**Figure S6A**), indicating that no intermolecular self-assembly of bacaucin-1a in aqueous solution. Besides, no intramolecular interaction of bacaucin-1a was observed through ^{1}H and ^{13}C NMR analysis (**Figure S6B, C**), suggesting that bacaucin-1a belongs to the family of extended/random-coil peptides which lack secondary structure in solution.

The method of screening spontaneous mutants has been successfully applied to reveal potential targets of novel antibiotics.[31] For example, telomycin is found to bind to bacterial phospholipid cardiolipin,[32] and humimycins specifically kills MRSA by inhibiting lipid II flippase.[33] Based on this approach, we evolved the wild-type *S. aureus* ATCC 29213 in the presence of sublethal levels of bacaucin-1a to obtain the mutants that resist to bacaucin-1a. After 90 days of continuous induction, we got the mutants that survive at 64 μg mL^{-1} bacaucin-1a (**Figure S7A, B**), which is 32 folds of MIC (2 μg mL^{-1}). However, the total protein expression in mutants shown the same patterns to that in the parent strain (**Figure S8**). Therefore, we further sequenced the whole-genomes of the mutants to gain a deep insight into the mode of action of bacaucin-1a. We identified four single-nucleotide polymorphisms (SNPs) not present in the wild-type one (**Table S7**). Each resistant isolate contained SNPs in GMP synthase and RNA polymerase sigma factor sigB (**Figure 3C, S7C**), confirmed by our optimized PCR tests and genetic sequencing analysis (**Table S8**). While, the other two SNPs were excluded as the potential targets of bacaucin-1a, due to the false-positive mutation by second generation sequencing. In *S. aureus*, GMP synthase participates in the *de novo* biosynthesis of purine nucleotides and glutamate metabolism.[34] Besides, RNA polymerase sigma factor sigB, by reversibly binding to the RNA polymerase,[35] is a master regulator for the stress response,[36] such as temperature tolerance and osmotic stress.[37,38] Combining the fact that the synergy of bacaucin-1a and rifampicin targeting RNA polymerases (**Figure S4, S5**), it suggests that GMP synthase and RNA polymerase sigma factor sigB may be the intracellular targets of bacaucin-1a. However, it remains unclear that whether these two intracellular targets are responsible for the high selectivity of bacaucin-1a against *S. aureus*.

To further investigate how bacaucin-1a reaches the intracellular targets, we evaluate the membrane integrity of *S. aureus* treated by bacaucin-1a. We first observed the collapsed and

lysed bacterial cells induced by bacaucin-1a by scanning electron microscope (SEM) (**Figure S9**). Meanwhile, the integrity of bacterial membrane was severely disrupted through LIVE/DEAD cell assays (**Figure S10A**). Additionally, increased membrane permeability was shown by the uptake of propidium iodide (PI) in dose and time dependent manners (**Figure S10B, C)**, which was consistent with the increased Ca^{2+} efflux (**Figure S10D**). These results suggest that bacaucin-1a may reach the cytosol of *S. aureus* through membrane disruption.

We next exploited the intracellular secondary responses of *S. aureus* treated by bacaucin-1a. Recent studies have shown that the stressful conditions may trigger an apoptotic-like death (ALD) mediated by *recA* and *lexA* genes or the *mazEF* pathway in bacteria.[39,40] Interestingly, we found that bacaucin-1a induced ten-fold increase of the expression of *recA* and *lexA* genes based on mRNA expression analysis, but not for the *mazEF* gene (**Figure 3D**). These results indicate that bacaucin-1a triggers an apoptotic-like death (ALD) pathway to induce programmed cell death in *S. aureus*. Meanwhile, dissipation of membrane potential and increased level of total reactive oxygen species (ROS) were observed as well (**Figure S10E, F**). Taken together, these results suggest that bacaucin-1a kills *S. aureus* through multiple strategies (**Figure 3E**).

Bacaucin-1a exhibits therapeutic efficacy in cellular and animal models. Given the attractive antibacterial activity of bacaucin-1a against MRSA *in vitro* and its unique mode of action, we further investigated its potential as a therapeutic agent in *in vitro* and *in vivo* models. Bacaucin-1a could effectively reduce the number of intracellular *S. aureus* in infected Vero cells (**Figure 4A, B**). Bacaucin-1a, similar to vancomycin, dramatically increased the survival rate and alleviated the pathologic changes of mice in the peritonitis-sepsis model (**Figure 4C, D, E and S11**). The infected mice without treatment were all died within 12 h. Collectively, these findings demonstrate the ability of bacaucin-1a against *S. aureus* associated infections at both cellular and *in vivo* levels.

Conclusions

In conclusion, our approach demonstrate that an alternative lead can be used to treat *S. aureus* associated infections. Unlike the classic cationic antimicrobial peptides, we rationally engineered

a short, linear, low cationic heptapeptide bacaucin-1a with remarkable antibacterial activity based on systematic structure-activity relationship study. Bacaucin-1a specifically kills MRSA in both *in vitro* and *in vivo* models. Our approach can be extended to other natural products to construct promising antibacterial agents for fighting antibiotic resistant bacteria.

Experimental section

Materials. All bacterial strains used in this study are listed in Table S2. Unless otherwise noted, strains were grown in brain heart infusion (BHI) broth or on BHI agar plates at 37 °C. Vero cells were grown in Modified Eagle's Medium (MEM, Gibco) supplemented with 1% heat inactivated fetal bovine serum (FBS, Invitrogen), 1% (w/v) penicillin-streptomycin and 1% (w/v) sodium pyruvate (Sigma-Aldrich). Ampicillin, colistin, ciprofloxacin, rifampicin, tetracycline, vancomycin and daptomycin were obtained from China Institute of Veterinary Drug Control.

Peptide synthesis. All derivatives of bacaucin-1 and bacaucin-1a were synthesized by GL Biochem (Shanghai, China). All the peptides (*C* to *N* terminals) were synthesized from natural amino acids using an Fmoc (9-fluorenylmethoxycarbonyl) strategy-based solid phase peptide synthesis method. All the detailed information of these derivatives can be found in Table S1.

Zeta potential measurement: The zeta-potential of bacaucin-1a was measured as bellow. Bacaucin-1a was first dissolved in distilled water to reach a final concentration of 128 μg/mL. After incubation at room temperature for 30 min, 1 mL of the solution was taken out for the surface potential measurement using Malvern Zetasizer (Nano-ZS). All the measurement was carried out at 25 °C.

Antibacterial tests. Minimum inhibitory concentrations (MICs) of bacaucin-1, its derivatives and other antibiotics were determined by broth micro-dilution according to the CLSI 2015 guideline.[41] Daptomycin was tested in the cation-adjusted Muller-Hinton broth with supplemented to 50 μg/mL calcium (Hope Bio Technology), while the others were tested in cation-adjusted Muller-Hinton broth (Land Bridge Technology). MIC values were defined as the lowest concentrations of antibiotics with no visible growth of bacteria after incubation at 37 °C for 16-20 h.

Stability of bacaucin-1a. The proteolytic resistance of bacaucin-1a and its derivatives were evaluated by treated with four kinds of proteinases (Sangon Biotech) including proteinase K, trypsin and papain which were dissolved in PBS (pH 7.0), while pepsin was dissolved in PBS (pH 2.0) at 37 °C for 1 h. To determine the stability of bacaucin-1a in the presence of serum,

bacaucin-1a was pre-treated with 50% fetal bovine serum at 37 °C for 12 h and the residual antimicrobial activity was determined. To determine the thermostability and pH stability, bacaucin-1a was treated in the temperature range from 20 °C to 121 °C for 1 h and in the pH range from 2.0 to 12.0 at 37 °C for 1 h, respectively. Subsequently, all samples were readjusted to pH 7.0 to determine the residual antimicrobial activity against *S. aureus* ATCC 29213.

Safety assessment. Hemolytic activity of the derivatives were determined according to a previous publication.[42] Briefly, sheep blood cells were prepared from fresh sterile defibrinated sheep blood (Land Bridge Technology) and treated with various concentrations of derivatives at 37 °C for 1 h, using PBS and 0.2% Triton X-100 as negative and positive controls, respectively. The absorption of released hemoglobin was measured at 576 nm. Cytotoxicity on mammalian cells was performed on Vero cells by water-soluble tetrazolium salt-1 (WST-1, Roche) assay.[43]

Cell membrane tests. Membrane permeability and dissipated membrane potential of *S. aureus* ATCC 29213 induced by bacaucin-1a were tested by 10 nmol/L propidium iodide (PI) and 3, 3-dipropylthiadicarbocyanine iodide ($DiSC_3$ (5)) (Aladdin). The concentration of intracellular Ca^{2+} was measured by fluorescent probe Fluo-3AM (Beyotime) after *S. aureus* ATCC 29213 treated by bacaucin-1a for 1 h. The total ROS of *S. aureus* ATCC 29213 in the presence or absence of bacaucin-1a was probed with 2',7'-dichlorofluor-escein diacetate (DCFH-DA). Rosup was used as the positive control for ROS production.

Screening bacaucin-1a-resistant mutants. Resistant *S. aureus* mutants were obtained through continuous passage culture. 1 to 10,000 diluted *S. aureus* ATCC 29213 overnight culture were inoculated in fresh MHB containing 0.25 × MICs, 0.5 × MICs, 1 × MICs and 2 × MICs and 4 × MICs of bacaucin-1a . The bacterial cultures were then incubated at 37 °C under continuous shaking at 200 rpm for 24 h.

Subsequently, the cultures from the second highest concentrations with visible growth (OD_{600} ≥ 2) were 1 to 100 diluted into a series of MHB media, until the presence of bacaucin-1a resistant mutants. Lastly, the culture that could grow at high MIC levels was passaged on antibiotic free BHI plates. Meanwhile, the correspondence MICs were determined as described above.

Whole genome sequencing (WGS) and SNPs (single-nucleotide polymorphisms) identification. Total DNA from *S. aureus* ATCC 29213 and bacaucin-1a resistant mutants (16 × MIC and 32 × MIC) were extracted using the Wizard Genomic DNA Purification kit (Promega). Then, the libraries were constructed using the NEXT Ultra DNA Library Prep kit (New England Biolabs) and 150 bp paired-end reads were sequenced by Illumina Hiseq 2500 (Annoroad). Lastly, the draft assembly of the sequences were generated using CLC Genomics Workbench 8 (CLC Bio).[44]

All MiSeq reads were mapped to the reference genome of *S. aureus* ATCC 29213 (RefSeq assembly accession: GCF_001879295.1) using BWA[45] for identification of the variants. Single-nucleotide polymorphisms (SNPs) were found in the bacaucin-1a resistant strains.

Bacteria-cell coculture. MRSA T144 were first stained by pHrodo Red (Life Technologies) for 45 min. Subsequently, Vero cells were infected with stained MRSA T144 cells at an MOI of 100, and co-culture with various bacaucin-1a concentrations for 5 hours at 37°C in 5% CO_2. After 50 μg/mL vancomycin treatment and PBS (phosphate buffered saline) washing, Vero cells were then fixed (4% paraformaldehyde), permeabilized (0.1% Triton X-100) and stained with actinGreen (Life Technologies) followed by Hoechst 33342 (Beyotime) staining. Lastly, fluorescent images were obtained by a confocal laser scanning microscope (Leica TCS SP8).

Mouse peritonitis-sepsis model. BALB/c female mice (n = 6 per group) were infected with 0.5 mL of MRSA T144 suspension (7.5×10^8 CFU per mouse) via intraperitoneal injection. At one hour post-infection, all mice were treated with bacaucin-1a at single intraperitoneal doses of 0, 2, 10, 20 and 40 mg/kg. Meanwhile, the other two groups were treated with vancomycin at doses of 2 and 20 mg/kg as positive controls. Survival rates of treated mice were recorded during 48 h.

Ethics statement. Mice were maintained in strict accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (11-14-1988). The animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the China

Agricultural University and were performed in accordance with the relevant guidelines and regulations (ID: SKLAB-B-2010-003). The laboratory animal usage license number is SYXK-2016-0008, certified by Beijing Association for Science and Technology. All animals were performed with 6-8 week old female BALB/c mice, purchased from the Vital River Laboratory (Beijing, China) and housed under specified pathogen-free condition for one week.

Associated content

In supporting information section, Figure S1 describes the antibacterial activity of *L*-alanine substitutions of bacaucin-1. Figure S2 and S3 provide the stability and safety evaluation of bacaucin-1a. Figure S4 and S5 show the synergistic activity of bacaucin-1a and other antibiotics. Figure S6 displays the ^{1}H NMR and ^{13}C NMR spectrum. Figure S7 and S8 describe the screening of bacaucin-1a resistant mutants and SDS-PAGE analysis of total protein expression. Figure S9 and S10 show the morphological changes and membrane dysfunctions of *S. aureus* by bacaucin-1a. Figure S11 provides the histopathological changes of mice organs in control, infected and treated groups. Table S1 and S2 show the chemical information of derivatives and bacterial strains used in this study. Table S3 and S4 describe the effect of conformation of amino acid and proteinase and serum on the antibacterial activity of bacaucin-1a. Table S5 and S6 show the antibacterial spectrum and activity of bacaucin-1a. Table S7 and S8 describe the SNPs in bacaucin-1a resistant mutants and primers used in this study.

Acknowledgements

The authors thank Prof. Wenhan Lin (Peking University) for detailed NMR analysis and Dr. Zhuojun Dai (Duke University) and Jianqi Wang (Chinese University of Hong Kong) for dynamic light scattering measurement. This work was supported by the National Key Research and Development Program of China (2017YFC1600305, 2018YFD0500506), National Natural Science Foundation of China (31772796), Beijing Dairy Industry Innovation Team and Beijing Municipal Science and Technology Project (D171100002017004).

Notes

The authors declare no competing financial interest.

References

(1) Kupferschmidt, K., (2016) Resistance fighter. *Science 352* (6287), 758-761. DOI: 10.1126/science.352.6287.758.

(2) Berendonk, T. U.; Manaia, C. M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Burgmann, H.; Sorum, H.; Norstrom, M.; Pons, M. N.; Kreuzinger, N.; Huovinen, P.; Stefani, S.; Schwartz, T.; Kisand, V.; Baquero, F.; Martinez, J. L., (2015) Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol. 13* (5), 310-317. DOI: 10.1038/nrmicro3439.

(3) Chambers, H. F.; DeLeo, F. R., (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol. 7* (9), 629-641. DOI:10.1038/nrmicro2200.

(4) Stokes, J. M.; MacNair, C. R.; Ilyas, B.; French, S.; Cote, J. P.; Bouwman, C.; Farha, M. A.; Sieron, A. O.; Whitfield, C.; Coombes, B. K.; Brown, E. D., (2017) Pentamidine sensitizes Gram-negative pathogens to antibiotics and overcomes acquired colistin resistance. *Nat. Microbiol. 2*, 17028. DOI: 10.1038/nmicrobiol.2017.28.

(5) Zipperer, A.; Konnerth, M. C.; Laux, C.; Berscheid, A.; Janek, D.; Weidenmaier, C.; Burian, M.; Schilling, N. A.; Slavetinsky, C.; Marschal, M.; Willmann, M.; Kalbacher, H.; Schittek, B.; Brotz-Oesterhelt, H.; Grond, S.; Peschel, A.; Krismer, B., (2016) Human commensals producing a novel antibiotic impair pathogen colonization. *Nature 535* (7613), 511-516. DOI: 10.1038/nature18634.

(6) Xiong, M.; Lee, M. W.; Mansbach, R. A.; Song, Z.; Bao, Y.; Peek, R. M., Jr.; Yao, C.; Chen, L. F.; Ferguson, A. L.; Wong, G. C.; Cheng, J., (2015) Helical antimicrobial polypeptides with radial amphiphilicity. *Proc. Natl. Acad. Sci. USA 112* (43), 13155-13160. DOI: 10.1073/pnas.1507893112.

(7) Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schaberle, T. F.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V. A.; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.; Zullo, A. M.; Chen, C.; Lewis, K., (2015) A new antibiotic kills pathogens without detectable resistance. *Nature 517* (7535), 455-459. DOI: 10.1038/nature14098.

(8) Hamamoto, H.; Urai, M.; Ishii, K.; Yasukawa, J.; Paudel, A.; Murai, M.; Kaji, T.; Kuranaga, T.; Hamase, K.; Katsu, T.; Su, J.; Adachi, T.; Uchida, R.; Tomoda, H.; Yamada, M.; Souma, M.; Kurihara, H.; Inoue, M.; Sekimizu, K., (2015) Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. *Nat. Chem. Biol. 11* (2), 127-133. DOI: 10.1038/nchembio.

(9) Schneider, T.; Kruse, T.; Wimmer, R.; Wiedemann, I.; Sass, V.; Pag, U.; Jansen, A.; Nielsen, A. K.; Mygind, P. H.; Raventós, D. S., (2010) Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II. *Science 328* (5982), 1168-1172. DOI: 10.1126/science.1185723.

(10) L.Fox, J., (2013) Antimicrobial peptides stage a comeback. *Nat. Biotechnol. 31* (5), 379-382. DOI: 10.1038/nbt.2572.

(11) Rajendran, L.; Knolker, H. J.; Simons, K., (2010) Subcellular targeting strategies for drug design and delivery. *Nat. Rev. Drug. Discov. 9* (1), 29-42. DOI: 10.1038/nrd2897.

(12) Wimley, W. C., (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chem. Biol. 5* (10), 905-917. DOI: 10.1021/cb1001558.

(13) Kohanski, M. A.; Dwyer, D. J.; Collins, J. J., (2010) How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol. 8* (6), 423-435. DOI:10.1038/nrmicro2333.

(14) Liu, Y.; Ding, S.; Shen, J.; Zhu, K., (2018) Nonribosomal antibacterial peptides that target multidrug-resistant bacteria. *Nat. Prod. Rep.* DOI: 10.1039/c8np00031j.

(15) Wenzel, M.; Chiriac, A. I.; Otto, A.; Zweytick, D.; May, C.; Schumacher, C.; Gust, R.; Albada, H. B.; Penkova, M.; Krämer, U., (2014) Small cationic antimicrobial peptides delocalize peripheral membrane proteins. *Proc. Natl. Acad. Sci. USA 111* (14), E1409-E1418. DOI: 10.1073/pnas.1319900111.

(16) Pai, M.; Neely, M.; Rodvold, K.; Lodise, T., (2014) Innovative approaches to optimizing the delivery of vancomycin in individual patients. *Adv. Drug Deliver. Rev. 77*, 50-57. DOI: 10.1016/j.addr.2014.05.016.

(17) Rabanal, F.; Cajal, Y., (2017) Recent advances and perspectives in the design and development of polymyxins. *Nat. Prod. Rep. 34* (7), 886-908. DOI: 10.1039/c7np00023e.

(18) Liu, Y.-Y.; Wang, Y.; Walsh, T. R.; Yi, L.-X.; Zhang, R.; Spencer, J.; Doi, Y.; Tian, G.; Dong, B.; Huang, X.; Yu, L.; Gu, D.; Ren, H.; Chen, X.; Lv, L.; He, D.; Zhou, H.; Liang, Z.; Liu, J.; Shen, J., (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis. 16*, 161-168. DOI: 10.1016/S1473-3099(15)00424-7.

(19) Yeung, A. T.; Gellatly, S. L.; Hancock, R. E., (2011) Multifunctional cationic host defence peptides and their clinical applications. *Cell. Mol. Life Sci. 68* (13), 2161-2176. DOI: 10.1007/s00018-011-0710-x.

(20) Easton, D. M.; Nijnik, A.; Mayer, M. L.; Hancock, R. E., (2009) Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol. 27* (10), 582-590. DOI: 10.1016/j.tibtech.2009.07.004.

(21) Fjell, C. D.; Hiss, J. A.; Hancock, R. E.; Schneider, G., (2012) Designing antimicrobial peptides: form follows function. *Nat. Rev. Drug. Discov. 11* (1), 37-51. DOI:10.1038/nrd3591.

(22) Hancock, R. E.; Sahl, H. G., (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol. 24* (12), 1551-1557. DOI:10.1038/nbt1267.

(23) Lam, S. J.; O'Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H.; Chen, Y. Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G., (2016) Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. *Nat. Microbiol. 1* (11), 16162. DOI: 10.1038/NMICROBIOL.2016.162.

(24) Li, P.; Zhou, C.; Rayatpisheh, S.; Ye, K.; Poon, Y. F.; Hammond, P. T.; Duan, H.; Chan-Park, M. B., (2012) Cationic peptidopolysaccharides show excellent broad-spectrum antimicrobial activities and high selectivity. *Adv. Mater. 24* (30), 4130-4137. DOI: 10.1002/adma.201104186.

(25) Mahlapuu, M.; Håkansson, J.; Ringstad, L.; Björn, C., (2016) Antimicrobial peptides: an emerging category of therapeutic agents. *Front. Cell. Infect. Microbiol. 6*, 194. DOI: 10.3389/fcimb.2016.00194.

(26) Kwon, E. J.; Skalak, M.; Bertucci, A.; Braun, G.; Ricci, F.; Ruoslahti, E.; Sailor, M. J.;

Bhatia, S. N., (2017) Porous silicon nanoparticle delivery of tandem peptide anti-infectives for the treatment of *Pseudomonas aeruginosa* lung infections. *Adv. Mater. 29* (35), 1701527. DOI: 10.1002/adma.201701527.

(27) Davis, S. A.; Vincent, B. M.; Endo, M. M.; Whitesell, L.; Marchillo, K.; Andes, D. R.; Lindquist, S.; Burke, M. D., (2015) Nontoxic antimicrobials that evade drug resistance. *Nat. Chem. Biol. 11* (7), 481-487. DOI: 10.1038/NCHEMBIO.1821.

(28) Cochrane, S.; Vederas, J., (2016) Lipopeptides from *Bacillus* and *Paenibacillus* spp.: a gold mine of antibiotic candidates. *Med. Res. Rev. 36* (1), 4-31. DOI: 10.1002/med.21321.

(29) Raaijmakers, J. M.; De Bruijn, I.; Nybroe, O.; Ongena, M., (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev. 34* (6), 1037-1062. DOI:10.1111/j.1574-6976.2010.00221.x.

(30) Liu, Y.; Ding, S.; Dietrich, R.; Märtlbauer, E.; Zhu, K., (2017) A biosurfactant-inspired heptapeptide with improved specificity to kill MRSA. *Angew. Chem. Int. Ed. 56* (6), 1486-1490. DOI:10.1002/anie.201609277.

(31) Farha, M. A.; Brown, E. D., (2016) Strategies for target identification of antimicrobial natural products. *Nat. Prod. Rep. 33* (5), 668-680. DOI: 10.1039/c5np00127g.

(32) Johnston, C. W.; Skinnider, M. A.; Dejong, C. A.; Rees, P. N.; Chen, G. M.; Walker, C. G.; French, S.; Brown, E. D.; Berdy, J.; Liu, D. Y.; Magarvey, N. A., (2016) Assembly and clustering of natural antibiotics guides target identification. *Nat. Chem. Biol. 12* (4), 233-239. DOI: 10.1038/NCHEMBIO.2018.

(33) Chu, J.; Vila-Farres, X.; Inoyama, D.; Ternei, M.; Cohen, L. J.; Gordon, E. A.; Reddy, B. V.; Charlop-Powers, Z.; Zebroski, H. A.; Gallardo-Macias, R.; Jaskowski, M.; Satish, S.; Park, S.; Perlin, D. S.; Freundlich, J. S.; Brady, S. F., (2016) Discovery of MRSA active antibiotics using primary sequence from the human microbiome. *Nat. Chem. Biol. 12* (12), 1004-1006. DOI: 10.1038/NCHEMBIO.2207.

(34) Reddy, B. A.; van der Knaap, J. A.; Bot, A. G.; Mohd-Sarip, A.; Dekkers, D. H.; Timmermans, M. A.; Martens, J. W.; Demmers, J. A.; Verrijzer, C. P., (2014) Nucleotide

biosynthetic enzyme GMP synthase is a TRIM21-controlled relay of p53 stabilization. *Mol. Cell 53* (3), 458-470. DOI: 10.1016/j.molcel.2013.12.017.

(35) Feklistov, A.; Darst, S. A., (2011) Structural basis for promoter-10 element recognition by the bacterial RNA polymerase σ subunit. *Cell 147* (6), 1257-1269. DOI: 10.1016/j.cell.2011.10.041.

(36) Tuchscherr, L.; Bischoff, M.; Lattar, S. M.; Noto Llana, M.; Pfortner, H.; Niemann, S.; Geraci, J.; Van de Vyver, H.; Fraunholz, M. J.; Cheung, A. L.; Herrmann, M.; Volker, U.; Sordelli, D. O.; Peters, G.; Loffler, B., (2015) Sigma factor SigB is crucial to mediate *Staphylococcus aureus* adaptation during chronic infections. *PLoS Pathog. 11* (4), e1004870. DOI: 10.1371/journal.ppat.1004870.

(37) Kaczmarzyk, D.; Anfelt, J.; Särnegrim, A.; Hudson, E. P., (2014) Overexpression of sigma factor SigB improves temperature and butanol tolerance of *Synechocystis* sp. PCC6803. *J. Biotechnol. 182*, 54-60. DOI: 10.1016/j.jbiotec.2014.04.017.

(38) Utratna, M.; Shaw, I.; Starr, E.; O'Byrne, C. P., (2011) Rapid, transient, and proportional activation of σ^B in response to osmotic stress in *Listeria monocytogenes*. *Appl. Environ. Microbiol. 77* (21), 7841-7845. DOI: 10.1128/AEM.05732-11.

(39) Erental, A.; Kalderon, Z.; Saada, A.; Smith, Y.; Engelberg-Kulka, H., (2014) Apoptosis-like death, an extreme SOS response in *Escherichia coli*. *mBio 5* (4), e01426-14. DOI: 10.1128/mBio.01426-14.

(40) Bayles, K. W., (2014) Bacterial programmed cell death: making sense of a paradox. *Nat. Rev. Microbiol 12* (1), 63. DOI: 10.1038/nrmicro3136.

(41) Clinical and Laboratory Standards Institute, (2015) Performance Standards for Antimicrobial Susceptibility Testing. *CLSI*.

(42) Chen, W. Y.; Chang, H. Y.; Lu, J. K.; Huang, Y. C.; Harroun, S. G.; Tseng, Y. T.; Li, Y. J.; Huang, C. C.; Chang, H. T., (2015) Self-assembly of antimicrobial peptides on gold nanodots: against multidrug-resistant bacteria and wound-healing application. *Adv. Funct. Mater. 25* (46), 7189-7199. DOI: 10.1002/adfm.201503248.

(43) Cui, Y.; Liu, Y.; Liu, X.; Xia, X.; Ding, S.; Zhu, K., (2016) Evaluation of the toxicity and toxicokinetics of cereulide from an emetic *Bacillus cereus* strain of milk origin. *Toxins (Basel) 8* (6), 156. DOI:10.3390/toxins8060156.

(44) Wang, Y.; Zhang, R.; Li, J.; Wu, Z.; Yin, W.; Schwarz, S.; Tyrrell, J. M.; Zheng, Y.; Wang, S.; Shen, Z.; Liu, Z.; Liu, J.; Lei, L.; Li, M.; Zhang, Q.; Wu, C.; Zhang, Q.; Wu, Y.; Walsh, T. R.; Shen, J., (2017) Comprehensive resistome analysis reveals the prevalence of NDM and MCR-1 in Chinese poultry production. *Nat. Microbiol. 2*, 16260. DOI: 10.1038/nmicrobiol.2016.260.

(45) Li, H.; Durbin, R., (2010) Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics 26* (5), 589-595. DOI: 10.1093/bioinformatics/btp698.

Figures

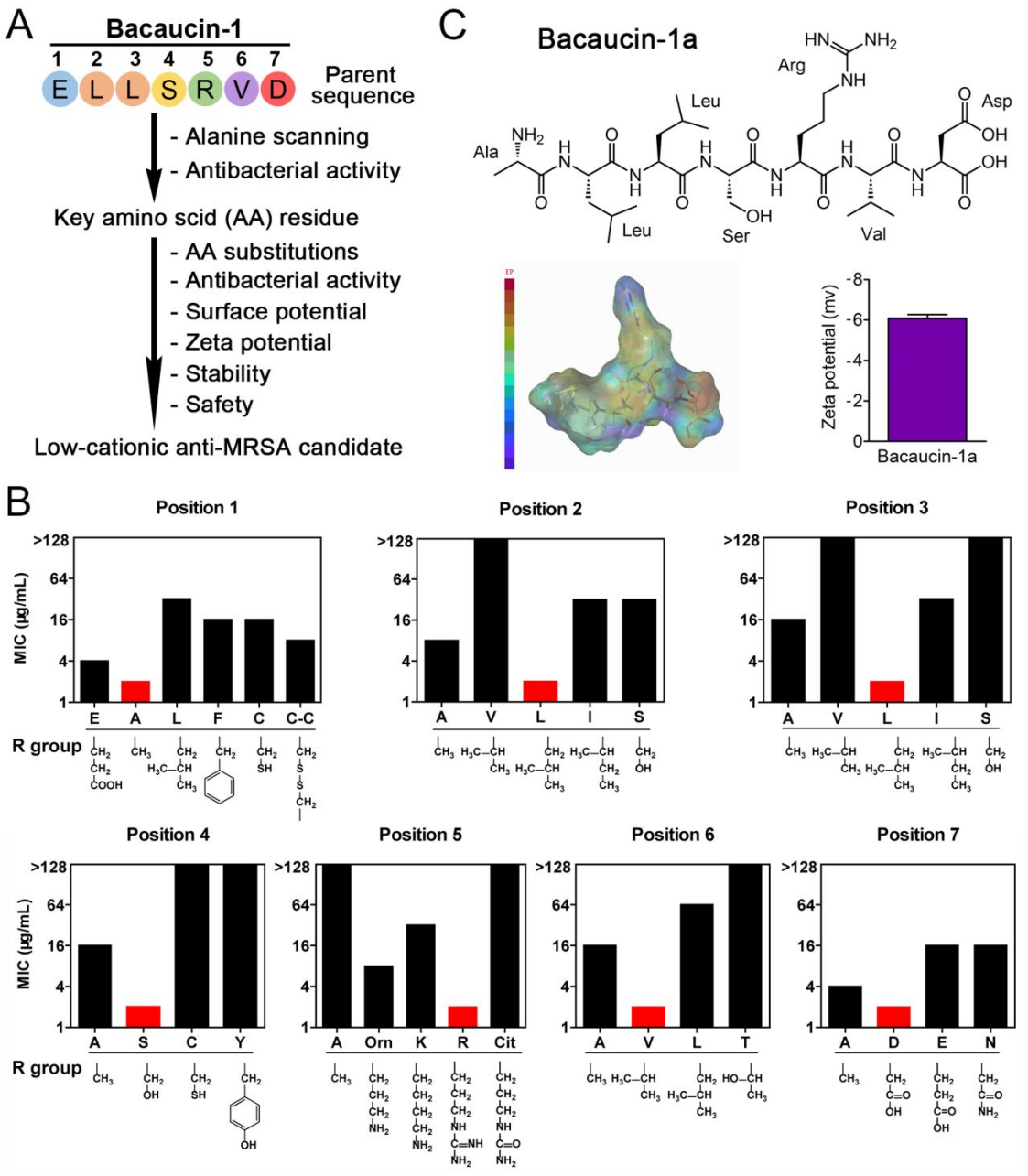

Figure 1 Structure-activity relationship (SAR) of bacaucin-1a.

(**A**) Flowchart of the screening progression of low-cationic peptide antibiotic in this study. (**B**) Antibacterial activity of bacaucin-1a derivatives. Each amino acid of bacaucin-1a was substituted for another one based on their polarities and electronic properties. The R groups (side chains) of each amino acid residue are shown. The amino acid compositions of bacaucin-1a were displayed in red column. Minimum inhibitory concentrations (MICs) are the lowest concentration of a synthesized peptide that inhibits the growth of *S. aureus* ATCC 29213 by broth micro-dilution method. All the peptides consists of *L*-type amino acids. See also the Supporting information

Table S1. (**C**) Chemical structure, surface potential and zeta potential of bacaucin-1a.

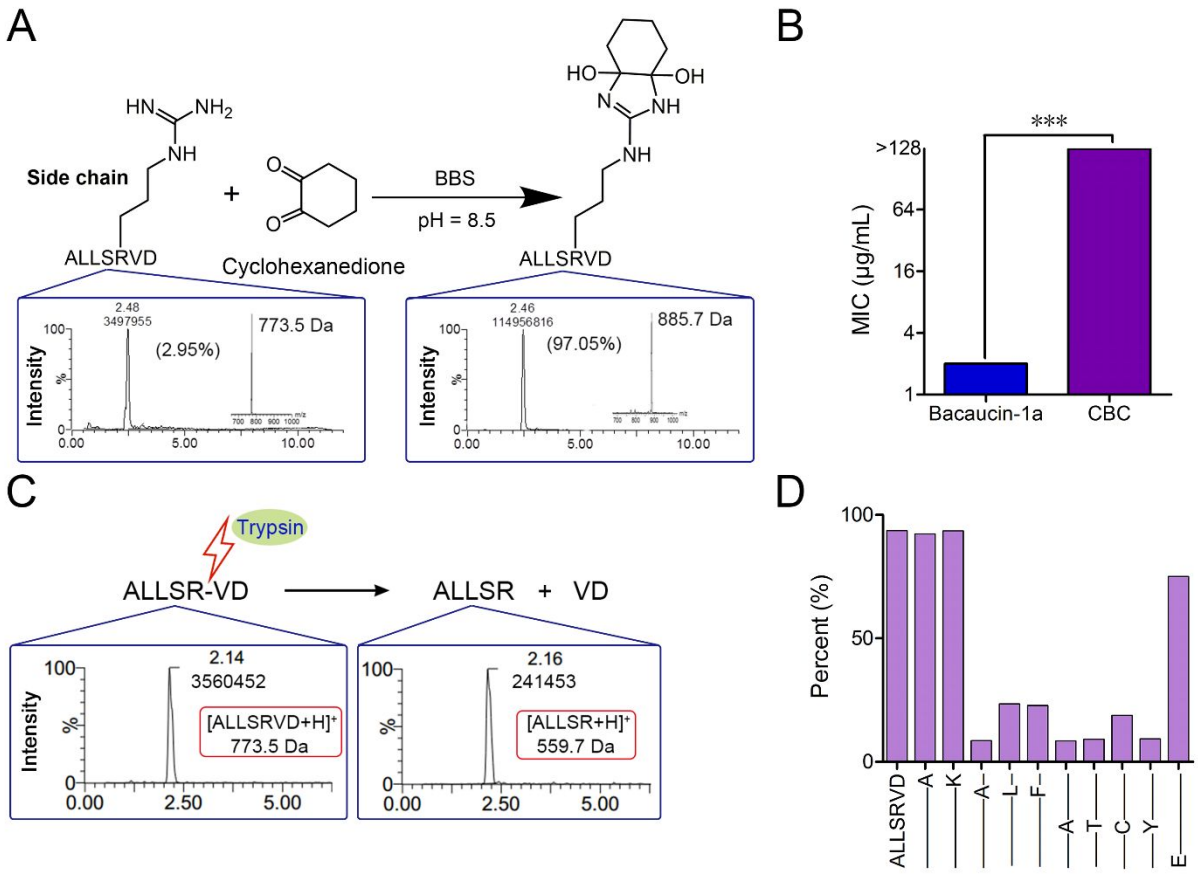

Figure 2 Active site and trypsin resistance of bacaucin-1a.
(**A**) Synthesis of 1,2-cyclohexanedione-bacaucin-1a conjugate (CBC) in borate buffered saline (BBS, pH = 8.5). The purity of CBC was determined by UPLC-MS/MS. (**B**) Antibacterial activity of bacaucin-1a and CBC against *S. aureus* ATCC 29213. Bacaucin-1a lost its antibacterial activity after guanidine group of arginine was conjugated. ***$P < 0.001$, determined by Student's *t* test. (**C**) Analysis of trypsin resistance in bacaucin-1a by UPLC-MS/MS. The retention times and corresponding pear areas of parent sequence and hydrolyzed product were shown. Percent (ALLSRVD, %) = 3560452 / (3560452 + 241453) = 93.65%, related to the first columns in Figure 2D. (**D**) Percentage of bacaucin-1a to the combination of bacaucin-1a and hydrolyzed product (ALLSR).

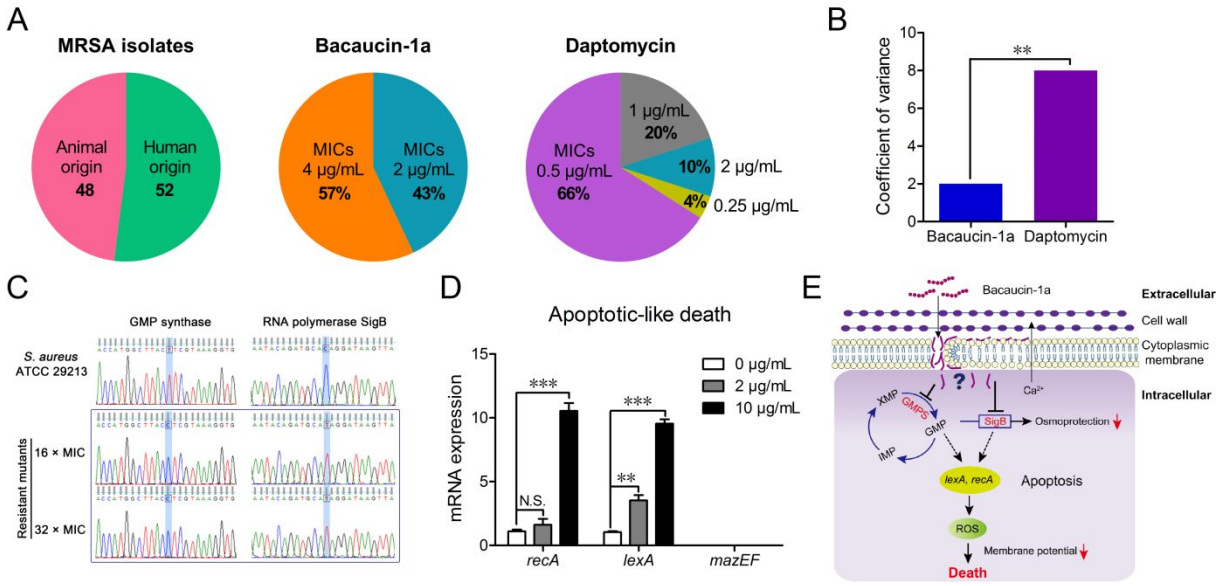

Figure 3 Mode of action of bacaucin-1a.

(**A**) Antibacterial activity of bacaucin-1a against 100 clinical MRSA isolates from both human and animal origins. (**B**) Bacaucin-1a displayed more stable antibacterial activity than daptomycin. Coefficient of variance represents the ratio of MIC_{max} to MIC_{min}. The smaller value of coefficient of variance, the more stable antibacterial activity. $^{**}P < 0.01$, determined by Student's *t* test. (**C**) The SNPs of GMP synthase and RNA polymerase SigB in bacaucin-1a resistant mutants. (**D**) Bacaucin-1a induced programmed cell death in *S. aureus* via the apoptotic-like death (ALD) pathway. N.S., not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, determined by non-parametric one-way ANOVA. (**E**) Schematic representation of mode of action of bacaucin-1a against *S. aureus*.

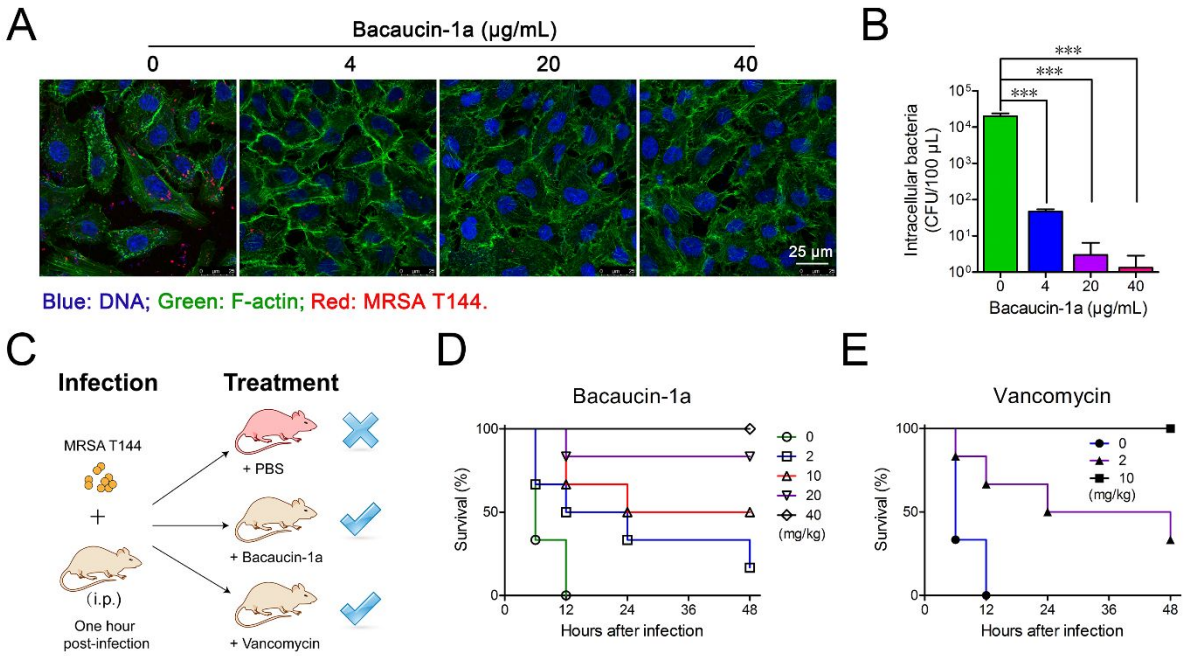

Figure 4 Bacaucin-1a was efficient in preventing infections in both cellular and *in vivo* models.

(A) Fluorescent micrographs of Vero cells infected with MRSA T144 in the presence of bacaucin-1a. DNA, F-actin and MRSA T144 were stained by hoechst, actin-green 488 and pHrodo red succinimidyl (NHS) ester, respectively. Scale bars, 25 μm. **(B)** Decreased numbers of the intracellular bacteria treated by bacaucin-1a. Data are represented as mean ± SD. ***$P <$ 0.001, determined by non-parametric one-way ANOVA. (**C**) Scheme of the experimental protocol for the mouse peritonitis-sepsis model. (**D and E**) Survival rates of the mice treated with bacaucin-1a (D) and vancomycin (E) infected by MRSA T144 (n = 6 per group).

Table of contents (TOC)