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Small Antimicrobial Agents Based on Acylated Reduced Amide Scaffold

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Abstract

Prevalence of drug-resistant bacteria has emerged to be one of the greatest threats in the 21st century. Herein, we report the development of a series of small molecular antibacterial agents that are based on the acylated reduced amide scaffold. These molecules display good potency against a panel of multidrug-resistant Gram-positive and Gram-negative bacterial strains. Meanwhile, they also effectively inhibit the biofilm formation. Mechanistic studies suggest that these compounds kill bacteria by compromising bacterial membranes, a mechanism analogous to that of host-defense peptides (HDPs). The mechanism is further supported by the fact that the lead compounds do not induce resistance in MRSA bacteria even after 14 passages. Lastly, we also demonstrate that these molecules have therapeutic potential by preventing inflammation caused by MRSA induced pneumonia in a rat model. This class of compounds could lead to an appealing class of antibiotic agents combating drug-resistant bacterial strains.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00640.

¹H NMR spectra, HPLC traces, and HR-MS results of compounds **1–16** and experimental details (PDF) Molecular formula strings and some data (CSV)

Notes

The authors declare no competing financial interest.

INTRODUCTION

Antibiotic resistance in bacteria is one of the most critical concerns faced in global public health.¹ Notorious multidrug-resistant strains, including Gram-positive bacteria methicillin-resistant *Staphylococcus epidermidis* (MRSE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci faecalis* (VRE), and Gram-negative bacteria *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, have emerged to be the major cause of hospital and community-acquired infections.^{2,3} As conventional antibiotics are observed to elicit escalating resistance, significant efforts have been extended to the investigation of host-defense peptides (HDPs) and their derivatives as alternative therapeutic strategies.^{4–6} Natural antimicrobial peptides, also known as HDPs, have attracted substantial interest in the past decade, and a few of them, such as PMX30063 and LTX109, are currently in clinical trials.^{4–7} HDPs are produced by living organisms as the first line of defense against a wide variety of infections.⁵ Unlike conventional antibiotics that target specific membrane or intracellular components of bacteria, HDPs preferentially interact with bacterial membranes and lead to the destruction of membranes eventually. The specificity of HDPs toward bacteria cells over mammalian cells lies on the intrinsic difference between bacterial and mammalian cell membranes. It is known that the outer leaflet of mammalian cell membranes comprises zwitterionic lipids such as cholesterol, phosphatidylcholine, and sphingomyelin. Their negatively charged phospholipids are generally sequestered in the inner leaflet of membranes.^{2,8} In contrast, negatively charged phospholipids including cardiolipin and phosphatidylglycerol are frequently identified in the outer leaflet of bacterial membranes.⁹ In addition, negatively charged molecules such as teichoic acids and lipoteichoic acids are membrane components of most Gram-positive bacteria, while lipopolysaccharides are characteristic molecules on the outer membranes of Gram-negative bacteria. As a result, bacterial membrane surfaces are generally more negatively charged than those of mammalian cells, leading to interaction with cationic HDPs preferentially. Although detailed antimicrobial mechanism of HDPs are still under debate, a few models including barrel stave, carpet, toroidal pores have been proposed, all of which more or less possess surfactants-like behavior.^{2,10} For instance, lipopeptides have been considered as the most potent natural biosurfactants.¹¹ As the interaction is based on physical charge—charge interaction and lacks defined membrane targets, it is believed that HDPs may decrease the risk of resistance development.¹² This is also why HDPs possess broad-spectrum

antibacterial activity and they are often bactericidal rather than bacteriostatic observed for most conventional antibiotics.² It should be noted that some HDPs do have defined intracellular targets including proteins, nucleic acids, and ribosomes; however, their cationic amphipathic structure is still crucial for their entry into bacterial cells, and membrane disruption may be the additional mechanism of action that further synergizes their antimicrobial activity.⁹

Nevertheless, HDPs face considerable challenges for further antibiotic development, among which low stability, moderate activity, and cost for production are major obstacles.¹³ Oligomeric peptidomimetics are resistant to proteolytic degradation, and through optimization, peptidomimetics such as peptoids,¹⁴ β -peptides,^{15–17} oligoureas,¹⁸ and AApeptides^{19–24} can mimic the structure and function of HDPs and show potent and broad-spectrum activity against multidrug-resistant bacterial pathogens. Thus, they hold promise for future development of new class of antimicrobial agents. However, their structural complexity and large molecular weights (>1500 Da) make the synthetic process tedious and time-consuming, significantly hindering the translation of HDPs into clinical applications. To overcome the drawbacks, considerable endeavors have been devoted to the development of small molecular mimetics of HDPs of simplicity. Examples include arylamide foldamers,²⁵ small peptoid mimics,^{26,27} β -amino acid derivatives,²⁸ dipeptide derivatives,²⁹ and aryl compounds.³⁰ In particular, it is exciting to witness that arylamide foldamers PMX series and dipeptide derivatives LTX compounds are currently in either phase II or phase III clinical trials (Figure 1), demonstrating the therapeutic potential of small HDP-mimicking antibiotic agents.

Reduced amide derivatives are peptides containing reduced amide bond and have been widely used as isosteric replacements for peptides (Figure 2).³¹ Even with one amide bond reduced to give secondary amine, the peptide sequences display significantly enhanced resistance to enzymatic degradation in contrast to canonical peptides.³¹ Recently there is growing interest in the exploration of biological application of reduced- amide-containing compounds, such as the use as enzyme inhibitors,³² analogues of endogenous peptides,³³ synthetic vaccines,³⁴ and DNA-complexing agents for gene delivery.³⁵ Several reduced-amide-containing sequences were also reported to mimic HDPs, as they are more resistant to proteolytic degradation.³⁶ Indeed, AApeptides as oligomeric acylated reduced-amides were shown to display broad-spectrum antibacterial activity.^{37,38} However, these reduced-amide-containing oligomers are fairly long and large (MW > 1800) sequences, and therefore their further optimization and potential for antibiotic therapeutics are still limited.

RESULTS AND DISCUSSION

To identify antibiotic agents with potentially more practical applications, herein we report the design and development of a class of small antimicrobial molecules containing acylated reduced amide (Figure 1). They are essentially built on a reduced dipeptide scaffold but with variable side chains. We hypothesized that if R_1 is a cationic group, while R_2 , R_3 , and R_4 are hydrophobic, the global structure of the molecules should be amphipathic, and thus they are expected to mimic HDPs and kill bacterial via membrane disruption. We anticipated that there are advantages associated with the molecular design. Reduced amide has been

demonstrated for their biological potential and stability. The design is also very straightforward and universal, as functional groups can be introduced very easily. Moreover, the activity of the molecules could be tuned by varying the hydrophobicity of R₂, R₃, and R₄ groups. As such, it is intriguing to investigate the antimicrobial activity of this class of small molecules and to evaluate their potential as antibiotic therapeutics.

The synthesis of these molecules was straightforward (Figure 3; see Supporting Information for details). Briefly, the building block³⁹ bearing the R₂ side chain was attached to chlorotrityl resin. After Fmoc group was removed, the R₃ moiety was attached to the secondary amine. Next, the alloc protecting group was removed, followed by capping with the R₁ group. The molecule was then cleaved from the solid support, to which the R₄ functionality was appended to the C-terminus to give the final product. We synthesized a series of such small molecules, and the structures of some lead compounds are shown in Table 1. Their antibacterial activity was also tested against a few clinically relevant bacteria, including multidrug resistant Gram-positive and Gram-negative bacterial pathogens (Table 1).^{23,40}

As shown in Table 1, with MWs ranging from 600 to 800, many molecules have shown good antibacterial activity against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria. The structure–activity relationship is also obvious in this class of molecules, allowing for their further optimization and development in the future. In compound **1**, R₁ and R₄ are hydrophobic naphthyl groups, while R₂ and R₃ are cationic groups. This compound did not show any antibacterial activity at the tested condition, possibly because the structure does not have segregated hydrophobic and cationic domains. We next kept R₁ as a cationic group and started to change the hydrophobicity of R₂, R₃, and R₄. As seen in compounds **2–4**, when R₂ is hydrophilic, the compounds were still not effective, albeit compound **2** exhibited some activity against all strains. This is likely due to the insufficient hydrophobicity of the molecules which do not endow the molecules with membrane activity. However, when R₂, R₃, and R₄ groups are all hydrophobic, compound **5** started to show activity against both Gram-positive and Gram-negative bacteria. It suggested that all R₂, R₃, and R₄ groups have to be hydrophobic so as to possess sufficient interactions with bacterial membranes. We also noticed that the guanidino cationic group is slightly weaker than the amino group in eliciting antimicrobial activity; thus we focused on the development of derivatives containing cationic amino groups. It was found that the antibacterial activity is highly related to the overall hydrophobicity of R₂, R₃, and R₄. Change of R₃ to adamantyl group seemed to significantly improve the antimicrobial activity of **7**, as its MIC against MRSE was 6.25 µg/mL already. When R₄ became more hydrophobic, for instance, being two phenyl groups, or biphenyl group, the activity of compounds **8** and **9** was further improved to be around 6.25 µg/mL for most of strains. Substitution on the aromatic group on R₄ was also likely to boost activity, as seen for **10**, **11**, and **12**, which contain the CF₃ or *tert*-butyl groups on the aromatic ring and displayed further enhanced activity. For instance, both **11** and **12** had the activity of less than 6.25 µg/mL for all tested strains. It should be noted that these compounds are quite selective, as their hemolytic activity are all more than 100 µg/mL. We hypothesized that the antibacterial activity could be further enhanced by increasing the hydrophobicity of R₄, and thus compound **13** was designed. This compound

contained adamantyl groups on both R₃ and R₄ positions, and as anticipated, it displayed more potent activity against MRSA with MIC of 1.56 $\mu\text{g/mL}$. It is known that hemolytic activity could be mitigated through the introduction of cationic charges.⁴³ We next designed the compound **14**, which contained one more lysine residue on the R₁ position compared with **13**. As expected, with one more cationic charge, compound **14** exhibited less hemolytic activity. However, the antibacterial activity of compound **14** was also less than **13**. Similar results were observed for **15** and **16** as well, suggesting that more lysine residues may not be preferred for bacterial killing. It is also interesting that with the replacement of lysine residue in **14** by an ornithine residue, compound **15** shows slightly increased hemolytic activity, even though the overall cationic charges remain the same. It may be because the side chain of lysine is slightly longer than that of ornithine, which decreases the potential nonspecific interactions. In addition, replacement of the lysine residue with arginine residue did not impact significantly either on antibacterial activity or on hemolytic activity, as seen for **14** and **16**, both of which show comparable selectivity toward bacteria. Next, we also tested the cytotoxicity of these compounds against HK-2 and K562 cells. Interestingly, although amphipathic agents are often cytotoxic, our compounds did not show noticeable cytotoxicity at the concentration of 25 $\mu\text{g/mL}$ against both HK-2 and K562 cells. The lead compound **13** has EC₅₀ of 86.3 and 83.2 $\mu\text{g/mL}$ against HK-2 and K562 cells, respectively, >50-fold of selectivity for MRSA.

As these acylated reduced-amide molecules were designed to possess global amphipathic structure of HDPs, we hypothesized they could mimic the mechanism of action of HDPs and exert their bactericidal activity by disrupting bacterial membranes. To evaluate the ability of these compounds to compromise bacterial membranes, fluorescence microscopy was used to visualize the impact of compounds on membranes of *S. aureus*.¹⁹ Although **11–13** have similar activity against the tested panel of bacteria, **13** and **14** were chosen in the study, as they show most potent activity in killing MRSA. Two dyes, 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Figure 4), were employed in the study. It is known that DAPI could stain membranes of bacterial cells with blue fluorescence irrespective of cell viability; however, the red fluorescence of PI due to the DNA intercalation could only be observed in the presence of impaired membranes that are permeable to PI. As shown in Figure 4, in the DAPI channel (460 nm), *S. aureus* species in the control groups are visible. However, they are not visible under the PI channel (620 nm). After bacteria were incubated with either **13** or **14**, they fluoresce under both PI and DAPI channels, suggesting the membranes of *S. aureus* were damaged.

It is known that membrane-active antibiotic agents could exert their bactericidal activity very rapidly, a phenomenon that has been widely observed in HDPs.⁴⁴ We thus next conducted time–kill study to investigate if **13** and **14** could rapidly kill MRSA in a dose-dependent fashion. Cell colonies were counted in agar plates at different time points at 4-, 8-, 16-fold of the MIC. As shown in Figure 5, compounds **13** and **14** at the concentration of either 25 or 50 $\mu\text{g/mL}$ could remove all bacteria completely in just 2 h. This observation indicates that **13** and **14** act in a similar manner to that of HDPs.

It is known that HDPs do not induce resistance in bacteria readily because they disintegrate bacterial membranes,⁴⁵ a mechanism lacking defined molecular targets. To evaluate the

probability of these reduced-amide-based small molecules to elicit resistance in bacteria, we carried out the drug resistance studies of **13** and **14** against MRSA. Their MICs toward MRSA were first measured, and then these compounds were incubated with bacteria in the well of the half-MIC and tested for their activity again. As shown in Figure 6, after 14 passages, MICs of both **13** and **14** are virtually very constant, indicating that these compounds do not induce drug resistance in MRSA, which suggests their mode of action is analogous to that of HDPs.

The biofilm is a notorious problem because bacteria in biofilm are generally more difficult to eradicate than planktonic cells. Thus, it could lead to severe chronic infectious diseases which complicate the treatment. In addition, the biofilm is also known to contaminate medical devices contributing to hospital acquired infections.⁴⁶ The biofilms formed by Gram-negative bacteria are even more challenging due to their stronger resistance. It is known that biofilms of *E. coli* and *A. baumannii* have posed great threat to community, and thus compounds that prevent their biofilm formation could lead to novel therapeutics.^{47,48} To evaluate the potential of the reduced- amide derived molecules in the biofilm prevention, compound **13** was evaluated for its ability to inhibit the biofilm formation of *E. coli* and *A. baumannii*. As shown in Figure 7, at just a concentration of 0.6 $\mu\text{g/mL}$, **13** could inhibit more than 50% of biofilm formation of both *E. coli* and *A. baumannii*. At the concentration of 2 $\mu\text{g/mL}$, it almost completely eradicated their biofilms.

Pneumonia is one of the most severe diseases caused by bacterial infection.⁴⁹ Community-acquired MRSA induced pneumonia has led to significant medicare cost and modality. Encouraged by the in vitro antibacterial profiles of our compounds, we tested their efficacy on a rat model bearing MRSA-induced ventilator-associated pneumonia⁵⁰ in order to evaluate their therapeutic potential. In this model anti-inflammatory activity is used as a surrogate for clearing the infection. The pathological analysis (Figure 8a) shows that local nasal administration of MRSA infection stimulated acute inflammation response in lungs, as indicated by the prevalence of neutrophils (blue spots in H&E staining). In the control group that was treated with saline, typical inflammation caused by pneumonia persisted over 3 days. In contrast, significant reduction of lung infiltrated proinflammatory cells could be observed after iv administration of compound **13** (10 mg/kg), strongly supporting the effectiveness of our antibacterial agents. To further support the observation, the level of the inflammatory cytokine TNF- α was also quantified by the ELISA assay. As shown in Figure 8b, the level of TNF- α cytokine increased over time in bronchoalveolar lavage fluid from the control rat group that received saline only, suggesting the persistence of lung inflammation. However, with the treatment of **13**, the cytokine level was lowered even after 1 day. At 3 days after treatment, the level of TNF- α level decreased, which was almost half of the level in control. The in vivo studies suggest that the compound **13** is capable of effectively suppressing the acute lung inflammation caused by pneumonia.

CONCLUSIONS

To summarize, we have developed a series of small antimicrobial agents based on the acylated reduced amide scaffold. These molecules display good potency against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria. Although other antimicrobial

mechanisms cannot be excluded, our mechanistic studies suggest that these compounds could kill bacteria rapidly by disrupting bacterial membranes, a mechanism analogous to that of host-defense peptides (HDPs). The hypothesis is further supported by the fact that the susceptibility of MRSA bacteria to the lead compounds remains nearly unchanged even after 14 passages. Meanwhile, they also exhibit high potency to inhibit the formation of biofilms. Furthermore, anti-inflammatory potential of these molecules was confirmed in the MRSA-induced pneumonia-bearing rat model. Although these types of agents have moderate to good selectivity so far, our work sheds light on the development of more potent compounds. Compared to HDPs, facile synthesis and high cost-effectiveness of these compounds make them appealing classes of antibiotic agents combating drug-resistant bacterial strains. Further studies on optimization of activity and selectivity, as well pharmacokinetic analysis, are underway in our lab.

EXPERIMENTAL SECTION

General Information

All Fmoc protected α -amino acids and Rink-amide resin (0.7 mmol/g, 200–400 mesh) were purchased from Chem-Impex International, Inc. Solvents and other reagents were purchased from either Sigma-Aldrich or Fisher Scientific and were used without further purification. Solid-phase synthesis of the compounds was carried out in the peptide synthesis vessel on a Burrell wrist-action shaker. The products were purified on a Waters Breeze 2 HPLC system, and lyophilized on a Labconco lyophilizer. The purity of the compounds was determined to be >95% by analytical HPLC (1 mL/min flow, 5–100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min was used). The NMR spectra were obtained on a Varian Inova 400 instrument.

Synthesis of Desired Compounds

Synthesis of **7** is shown below. The other compounds were synthesized following the similar procedure of **7**. 2-Chlorotrityl chloride (CTC) resin (200 mg, 0.2 mmol) was swelled in 3 mL of DCM for 15 min. The attachment of the first building block to the resin was achieved by adding γ -BB-1 (154 mg, 0.3 mmol) and *N,N*-diisopropylethylamine (DIPEA) (173 μ L, 1 mmol) to the beads in the reaction vessel, which was allowed to shake at room temperature for 2 h. After that, the reaction solution was drained, followed by washing with DMF (2 mL $\times$ 3) and DCM (2 mL $\times$ 3). The unreacted 2-chlorotrityl chloride moieties were capped with 3 mL of methanol for 30 min. The beads were washed with DCM (3 mL $\times$ 3) and DMF (3 mL $\times$ 3). The Fmoc group was removed by treating beads with 20% piperidine/DMF (v/v) solution for 10 min ($\times$ 2) at room temperature. The solution was drained and washed with DMF (3 mL $\times$ 3) and DCM (3 mL $\times$ 3). The beads were reacted with 1-admantaneacetyl chloride (72 μ L, 0.4 mmol) and DIPEA (87 μ L, 0.5 mmol) for 30 min ($\times$ 2) at room temperature, and the solution was removed. After DMF (2 mL $\times$ 3) and DCM (2 mL $\times$ 3) wash, the beads were treated with Pd(PPh₃)₄ (24 mg, 0.02 mmol) and Me₂NH $\dot{C}$ BH₃ (70 mg, 1.2 mmol) in 2 mL of DCM for 10 min ($\times$ 2) to remove the alloc protecting group. After the reaction, the solution was drained, and the beads were washed with DCM (3 mL $\times$ 3) and DMF (3 mL $\times$ 3). Next, Fmoc-Lys(Boc)-OH (187 mg, 0.4 mmol), *N,N*-diisopropylcarbodiimide (DIC) (84 μ L, 0.8 mmol), and hydroxybenzotriazole (HOBt) (122

mg, 0.8 mmol) were premixed in 2 mL of DMF for 5 min before getting transferred to the reaction vessel. The reaction was shaken at room temperature for 3 h, and the solution was drained. Then the intermediate was cleaved from resin with 5 mL of the cleavage cocktail (acetic acid/2,2,2-trifluoroethanol/DCM 1:1:8, v/v/v) for 2 h. The solution was collected, and the remaining beads were washed with 3 mL of the cleavage cocktail solution three times. All the solution was combined and concentrated in vacuo with coevaporation with hexane multiple times to completely remove acetic acid. A white solid intermediate was obtained. This intermediate was premixed with HOBt and DIC in DMF for 5 min, followed by the addition of 1-naphthylmethylamine. The clear faint yellow solution was stirred at room temperature for 5 h, then water was added to quench the reaction, followed by ethyl acetate (EtOAc) extraction. The organic layer was washed with 1 M HCl (×3) then brine (×1), dried over anhydrous Na₂SO₄, and concentrated by vacuum. The residue was treated with 50% TFA in DCM for 2 h, and then the solvent was removed with the N₂ flow. The residue was then treated with 50% diethylamine in CH₃CN for 30 min to remove Fmoc protecting group, followed by the removal of the solvent. The crude was analyzed and prepared on Waters HPLC system, followed by lyophilization to give the pure product 7.

Compound 1

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.26–8.65 (m, 2H), 7.80–8.03 (m, 5H), 7.54–7.79 (m, 6H), 7.37–7.53 (m, 3H), 4.60–4.74 (m, 1.5H), 4.23–4.28 (m, 0.8H), 3.85–4.13 (m, 2H), 3.48–3.66 (brd m, overlapped with H₂O, 4.5H), 3.41 (dd, *J* = 14.8, 9.2 Hz, 0.6H), 3.26 (dd, *J* = 14.8, 9.2 Hz, 0.5H), 2.61–2.75 (m, 3H), 2.50–2.59 (m, 1H), 2.12–2.80 (m, 0.8H), 1.26–1.75 (m, 6H). HRMS (ESI) C₃₄H₄₂N₅O₃ [M + H]⁺ calcd = 568.3282; found = 568.3271.

Compound 2

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.99 (t, *J* = 5.6 Hz, 0.3H), 8.47–8.52 (m, 1H), 8.10 (brd, 2H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.90 (s, 1H), 7.81 (t, *J* = 7.2 Hz, 2H), 7.77 (brd, 1H), 7.69 (d, *J* = 8.0 Hz, 1.3H), 7.42–7.59 (m, 5H), 7.28 (t, *J* = 7.2 Hz, 0.8H), 7.15 (d, *J* = 6.4 Hz, 0.7H), 4.71–4.83 (m, 0.8H), 4.68 (brd, 1.4H), 4.00–4.39 (m, 2.6H), 3.69 (overlapped, 2H), 2.74–2.83 (m, 2H), 1.21–1.60 (m, 6H). HRMS (ESI) C₃₀H₃₅N₄O₂ [M + H]⁺ calcd = 483.2755; found = 483.2756.

Compound 3

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.59 (t, *J* = 5.6 Hz, 0.4H), 8.36–8.41 (m, 1H), 8.22 (d, *J* = 8.8 Hz, 0.6H), 8.14 (brd, 2H), 8.03 (dd, *J* = 6.4, 2.4 Hz, 1H), 7.93 (td, *J* = 5.6, 1.8 Hz, 1H), 7.80–7.86 (m, 1.8H), 7.6 (brd, 4H), 7.39–7.54 (m, 3.5H), 4.73 (dd, *J* = 12.8, 5.6 Hz, 1.8H), 4.12 (d, *J* = 15.2 Hz, 0.5H), 3.93–4.08 (m, 2H), 3.63–3.72 (m, 3H), 3.32 (dd, *J* = 15.2, 4.8 Hz, 1H), 3.08 (dd, *J* = 13.6, 8.0 Hz, 0.6H), 2.70–2.73 (m, 3H), 2.40 (d, *J* = 13.6 Hz, 1H), 1.72–1.93 (m, 4H), 1.47–1.63 (m, 18H), 1.13–1.35 (m, 5H). HRMS (ESI) C₃₇H₅₇N₆O₃ [M + H]⁺ calcd = 633.4487; found = 633.4485.

Compound 4

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.34–8.64 (m, 2H), 8.19 (brs, 2.8H), 7.90–7.99 (m, 3.5H), 7.70–7.81 (m, 8H), 7.41–7.58 (m, 4H), 7.26–7.32 (m, 1H), 7.16 (d, *J* = 6.8 Hz, 0.6H),

4.78 (brd, 0.5H), 4.63 (d, $J = 5.2$ Hz, 1.3H), 4.10–4.25 (m, 1.4H), 3.96 (brd, 2H), 3.17 (overlapped, 2H), 3.46–4.39 (m, 2H), 3.24–3.30 (m, 0.6H), 2.56–2.74 (m, 4.4H), 1.51–1.74 (m, 5H), 1.20–1.42 (m, 6H), 1.00–1.14 (m, 1H). HRMS (ESI) $C_{36}H_{46}N_6NaO_3$ $[M + Na]^+$ calcd = 633.3524; found = 633.3636.

Compound 5

1H NMR (400 MHz, DMSO- d_6) δ 8.66 (t, $J = 4.4$ Hz, 0.3), 8.44–8.66 (m, 1.5H), 8.16 (brd, 2H), 7.96 (t, $J = 8.0$ Hz, 3H), 7.85 (t, $J = 8.0$ Hz, 2H), 7.76 (brd, 2H), 7.43–7.63 (m, 4.8H), 7.18–7.36 (m, 5H), 6.86–7.09 (m, 1H), 4.47–4.83 (m, 3.8H), 4.12–4.16 (m, 2.8H), 3.96–4.06 (m, 1.6H), 3.06–3.76 (m, 2.5H), 2.78–2.92 (m, 1.5H), 2.56–2.67 (m, 1.7H), 1.31–1.78 (m, 6H). HRMS (ESI) $C_{39}H_{44}N_5O_3$ $[M + H]^+$ calcd = 630.3439; found = 630.3424.

Compound 6

1H NMR (400 MHz, DMSO- d_6) δ 8.37–8.62 (m, 1.6H), 8.10–8.21 (m, 3H), 7.90–7.97 (m, 3H), 7.80–7.84 (m, 1.6H), 7.66–7.73 (m, 1H), 7.37–7.58 (m, 5H), 7.15–7.32 (m, 6.6H), 6.83–7.01 (m, 1.7H), 4.80–4.92 (m, 0.5H), 4.63–4.68 (m, 1H), 4.39–4.46 (m, 1H), 4.12–4.30 (m, 2H), 3.99 (d, $J = 17.2$ Hz, 2H), 3.82 (dd, $J = 13.6, 6.4$ Hz, 0.6H), 3.65–3.69 (m, 1H), 3.39–3.55 (m, 1H), 2.78–3.07 (m, 2H), 2.51–3.07 (m, 0.6H), 0.87–1.73 (m, 4H). HRMS (ESI) $C_{39}H_{44}N_7O_3$ $[M + H]^+$ calcd = 658.3500; found = 658.3496.

Compound 7

1H NMR (400 MHz, DMSO- d_6) δ 8.57–8.63 (m, 1H), 8.32–8.34 (m, 1H), 8.01–8.09 (m, 3H), 7.92 (dd, $J = 9.6, 6.0$ Hz, 1H), 7.78–1.85 (m, 3H), 7.40–7.53 (m, 4H), 7.14–7.30 (m, 4H), 4.68–4.77 (m, 2H), 4.07–4.21 (m, 2H), 3.83–4.02 (m, 4.8H), 3.66–3.69 (m, 0.9H), 3.47–3.58 (m, 1.2H), 3.37–3.48 (m, 1H), 3.15 (dd, $J = 13.6, 7.6$ Hz, 0.5H), 2.65–3.91 (m, 3.4H), 2.04–2.15 (m, 0.5H), 1.74–1.92 (m, 4H), 1.45–1.63 (m, 14H), 1.19–1.36 (m, 2.8H). HRMS (ESI) $C_{40}H_{54}N_5O_3$ $[M + H]^+$ calcd = 652.4221; found = 652.4204.

Compound 8

1H NMR (400 MHz, DMSO- d_6) δ 8.57 (d, $J = 8.8$ Hz, 0.5H), 8.31 (d, $J = 8.8$ Hz, 0.5H), 8.05 (brd, 2H), 7.77 (brd, 2H), 7.15–7.38 (m, 16H), 4.61–4.67 (m, 0.5H), 4.25–4.57 (m, 5H), 4.13–4.18 (m, 1H), 4.00–4.08 (m, 1H), 3.47–3.64 (m, 4H), 3.12 (dd, $J = 9.6, 7.2$ Hz, 0.6H), 2.62–2.78 (m, 4H), 2.25 (d, $J = 13.6$ Hz, 0.6H), 1.76–1.86 (m, 3H), 1.52–1.72 (m, 10H), 1.38–1.47 (m, 3H), 1.11–1.35 (m, 6H). HRMS (ESI) $C_{43}H_{58}N_5O_3$ $[M + H]^+$ calcd = 692.4534; found = 692.4517.

Compound 9

1H NMR (400 MHz, DMSO- d_6) δ 8.51–8.61 (m, 0.8H), 8.29–8.33 (m, 1H), 8.01–8.09 (m, 2.3H), 7.75 (brd, 2H), 7.54–7.61 (m, 3H), 7.42 (t, $J = 7.2$ Hz, 2H), 7.14–7.34 (m, 7H), 4.09–4.31 (m, 3H), 3.83–4.03 (m, 2H), 3.67 (m, 2H), 3.36–3.44 (m, 2H), 2.17 (dd, $J = 13.6, 8.0$ Hz, 0.6H), 2.51–2.78 (m, 4H), 1.76–2.13 (m, 4H), 1.43–1.61 (m, 15H), 1.18–1.19 (m, 2H). HRMS (ESI) $C_{42}H_{56}N_5O_3$ $[M + H]^+$ calcd = 678.4378; found = 678.4365.

Compound 10

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.52–8.70 (m, 1H), 8.31–8.42 (m, 1H), 8.05–8.09 (brdm, 2H), 7.75 (m, 2H), 7.72 (dd, *J* = 20.0, 8.0 Hz, 2H), 7.43–7.48 (m, 2H), 7.12–7.30 (m, 5H), 4.31–4.32 (m, 2H), 4.09–4.21 (m, 1H), 3.81–4.03 (m, 2H), 3.66–3.68 (m, 1H), 3.36–3.43 (m, 2H), 3.14 (dd, *J* = 13.6, 8.0 Hz, 0.5H), 2.51–2.78 (m, 4H), 2.11 (d, *J* = 13.6 Hz, 0.5H), 1.75–1.93 (m, 4H), 1.47–1.56 (brdm, 15H), 1.12–1.29 (m, 2H). HRMS (ESI) C₃₇H₅₁F₃N₅O₃ [M + H]⁺ calcd = 670.3939; found = 670.3930.

Compound 11

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.43–8.57 (m, 1H), 8.31 (d, *J* = 12.4 Hz, 0.4H), 8.21 (t, *J* = 5.6 Hz, 0.6H), 7.99–8.09 (brdm, 2.7H), 7.76 (brd, 2H), 7.12–7.35 (m, 8H), 4.14–4.22 (m, 3H), 3.80–4.05 (m, 3H), 3.65 (m, 1H), 3.35–3.53 (m, 3H), 3.16 (dd, *J* = 13.6, 8.0 Hz, 0.5H), 2.51–2.91 (m, 4H), 2.10 (d, *J* = 13.6 Hz, 0.6H), 1.72–1.94 (m, 4H), 1.50–1.58 (brdm, 14H), 1.07–1.37 (m, 12H). HRMS (ESI) C₄₀H₆₀N₅O₃ [M + H]⁺ calcd = 658.4691; found = 658.4680.

Compound 12

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.77 (dd, *J* = 12.0, 6.0 Hz, 0.4H), 8.54 (d, *J* = 8.8 Hz, 0.4H), 8.47 (t, *J* = 6.0 Hz, 0.6H), 8.31 (d, *J* = 8.4 Hz, 0.4H), 8.04–8.08 (brdm, 2.7H), 7.97 (s, 0.6H), 7.93 (brs, 2H), 7.75 (brd, 2H), 7.14–7.29 (m, 4.6H), 4.41–4.46 (m, 2H), 4.15–4.20 (m, 1H), 3.81–4.10 (m, 3H), 3.59 (m, 1H), 3.35–3.53 (m, 2.6H), 3.13 (dd, *J* = 13.6, 8.0 Hz, 0.4H), 2.51–2.85 (m, 4H), 2.08 (d, *J* = 13.6 Hz, 0.5H), 1.63–1.87 (m, 5H), 1.49–1.58 (brdm, 14H), 1.20–1.31 (m, 2H). HRMS (ESI) C₃₈H₅₀F₆N₅O₃ [M + H]⁺ calcd = 738.3812; found = 738.3815.

Compound 13

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.57 (t, *J* = 8.4 Hz, 0.4H), 8.34 (t, *J* = 8.4 Hz, 0.5H), 8.08 (brd, 2H), 7.89–7.94 (m, 0.6H), 7.78 (brd, 2H), 7.55–7.61 (m, 0.4), 7.18–7.27 (m, 4H), 4.12–4.22 (m, 1H), 3.93–4.00 (m, 1.3H), 3.62–3.78 (m, 3.6H), 3.15–3.22 (m, 0.7H), 2.73 (brd, 5.3H), 2.42–2.55 (m, 2.2H), 2.13–2.18 (m, 0.3 H), 1.75–1.92 (m, 7H), 1.52–1.60 (brd, 20H), 1.40 (brd, 7H), 1.20–1.27 (m, 2H). HRMS (ESI) C₄₀H₆₂N₅O₃ [M + H]⁺ calcd = 660.4847; found = 660.4855.

Compound 14

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.38–8.48 (m, 1H), 8.14 (brd, 2H), 7.88–7.99 (m, 1.4H), 7.82 (brd, 3H), 7.51–7.57 (m, 0.4H), 7.11–7.25 (m, 4.6H), 3.95–3.18 (m, 3H), 3.67–3.76 (m, 1.5H), 3.51–3.52 (m, 2H), 3.33–3.37 (m, 2H), 3.02–3.12 (m, 0.5H), 2.51–2.84 (m, 8H), 2.20 (dd, *J* = 13.6, 8.0 Hz, 0.4H), 1.79–1.97 (m, 7H), 1.44–1.64 (brdm, 23H), 1.20–1.40 (m, 11H), 0.94 (dd, *J* = 14.8, 7.2 Hz, 1H). HRMS (ESI) C₄₆H₇₄N₇O₄ [M + H]⁺ calcd = 788.5797; found = 788.5797.

Compound 15

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.50–8.66 (m, 1H), 8.20 (brd, 2H), 7.95–8.18 (m, 1.8H), 7.80–7.88 (brd, 3H), 7.51–7.62 (m, 0.5H), 7.11–7.27 (m, 4.7H), 3.90–4.18 (m, 7H), 3.52–

3.82 (m, 2.6H), 3.37–3.41 (m, 1H), 2.96–3.96 (m, 0.4H), 2.52–2.87 (m, 8H), 1.78–2.18 (m, 8H), 1.30–1.69 (brdm, 31H), 0.80–0.93 (m, 1H). HRMS (ESI) $C_{45}H_{72}N_7O_4$ $[M + H]^+$ calcd = 774.5640; found = 774.5651.

Compound 16

1H NMR (400 MHz, DMSO- d_6) δ 8.43–8.51 (m, 1H), 8.15–8.21 (m, 0.8H), 8.10 (brd, 2H), 7.86–8.02 (m, 1.2H), 7.73 (brd, 2H), 7.48–7.63 (m, 1.2H), 7.14–7.24 (m, 5H), 3.90–4.23 (m, 3.5H), 3.72–3.76 (m, 1.5H), 3.50–3.55 (m, 3H), 2.87–3.15 (m, 3H), 2.57–2.79 (m, 6H), 1.82–1.95 (m, 7H), 1.10–1.59 (m, 33H). HRMS (ESI) $C_{46}H_{74}N_9O_4$ $[M + H]^+$ calcd = 816.5858; found = 816.5866.

Minimum Inhibitory Concentrations (MICs) against Bacteria

The antimicrobial activity of the compounds was tested on the following six bacteria strains: *K. pneumoniae* (ATCC 13383), *P. aeruginosa* (ATCC27853), *E. coli* (ATCC 25922), vancomycin-resistant *Enterococcus faecalis* (ATCC 700802), methicillin-resistant *S. aureus* (MRSA, ATCC 33591), methicillin-resistant *S. epidermidis* (MRSE, RP62A). The procedures were followed as reported previously.¹⁹ The MICs were determined as the lowest concentration that completely inhibits the bacteria growth. The experiments were repeated at least three times with duplicates each time.

Hemolytic Assays

The freshly drawn, K2EDTA treated human red blood cells (hRBCs) were washed with 1× PBS buffer and centrifuged at 3500 rpm for 10 min. The step was repeated until the supernatant became clear. The supernatant was removed, and the RBCs were diluted into 5% v/v suspension. 50 μ L of the suspension was incubated with 50 μ L of compounds of different concentrations at 37 °C for 1 h. The mixture was centrifuged at 3500 rpm for 10 min. Subsequently, to 30 μ L of the supernatant, 100 μ L of PBS was added, and the absorbance of the mixture at 540 nm was read on a Biotek Synergy HT plate reader. The positive control was 2% Triton X-100, and the negative control was 1× PBS alone. The hemolysis activity was calculated by the formula % hemolysis = $[(Abs_{sample} - Abs_{PBS}) / (Abs_{Triton} - Abs_{PBS})] \times 100$. The experiment was repeated at least three times with duplicates each time.

MTT Assays

Both HK-2 and K562 cells were used to access the cell viability with treatment of compounds **5–16** using standard procedure.²² Cells were seeded in 96-well plate with 5×10^4 cells in 100 μ L of media per well. Control and blank wells were prepared accordingly. Serial dilutions of compounds at concentrations of 100, 50, 25, 12.5, 6.25 μ g/mL were prepared by diluting stock solution with media. After incubation for 12 h, 100 μ L aliquots of drugs were added and the plate was incubated for 24 h. All media in cells were then removed and washed with fresh media once, followed by addition of 110 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent and then incubation for another 4 h, after which 100 μ L of prewarmed solubilization solution was added. The plate was then incubated at 37 °C for 12 h before absorbance was read at 550 nm. The data

were calculated based on the following equation: Cell viability % = $[(A - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})] \times 100$. The measurements were repeated at least three times.

Fluorescence Microscopy

Fluorescence microscopy was used to assess the ability of the compounds to compromise bacterial membranes. Fluorescent dyes propidium iodide (**PI**) and 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) were used in the study. DAPI stains bacteria cells irrespective of their viabilities, whereas PI can only permeate bacterial cells and intercalate DNA in order to fluoresce when bacterial cell membranes are damaged membranes. After MRSA grew to mid-logarithmic phase, **13** or **14** was incubated with the bacteria at 37 °C for 2 h. The mixture was then centrifuged at 5000 rpm for 15 min. The cell pellets were washed with the PBS buffer three times and incubated with **PI** (5 µg/mL) and DAPI (10 µg/mL) for 15 min sequentially on ice in the dark. The mixture was then centrifuged, and the pellets were washed with the PBS buffer. Next, an amount of 10 µL of the samples was placed on chamber slides and observed under Zeiss Axio Imager ZI optical microscope using 100×oil-immersion objective.

Time-Kill Study

The kinetics of bacteria killing by the lead compounds **13** and **14** were investigated. Briefly, the bacteria MRSA grew to mid-logarithmic phase in tryptic soy broth (TSB) medium, from which the suspension (10^6 colony-forming units per milliliter (CFU/mL)) was made. The suspension was incubated with different concentrations of **13** or **14** for 10 min, 30 min, 1 h, and 2 h, respectively. The mixtures were diluted by 10^2 - to 10^4 -fold and spread on TSB agar plates. After incubation at 37 °C overnight, the colonies on the plates were counted and plotted against the incubation time.

Drug Resistance Study

The initial MICs of compounds **13** and **14** for MRSA were obtained as above. Bacteria in wells containing a concentration of $1/2$ MIC were used to make bacterial suspension (10^6 CFU/mL) for the next measurement of MICs of **13** and **14**. The experiment was repeated each day for 14 passages.

Inhibition of Biofilms

Overnight grown bacteria were inoculated into fresh 10% of MHII broth at a ratio of 1:100. 100 µL of inoculated culture was incubated with an appropriate amount of compound **13** in wells of 96-well plate. The plate was incubated at 37 °C overnight. Optical density of each well was recorded at a wavelength of 600 nm, and then the biofilm biomass was recorded by the crystal violet method. Biofilm biomass was presented as CV OD/OD of growth. Relative biofilm biomass values were normalized by the biomass value of control (no addition of compound). Experiments were conducted in triplicate, and the data were presented as the mean ± STDEV.

In Vivo Study. Establishment of the Rat Model of MRSA- Induced Pneumonia

All animal experiments were approved by the Institutional Committee for Animal Care of Nanjing University and carried out in accordance to the policy of the National Ministry of Health of China. Briefly, male Wistar rats (6–8 weeks, average weight 200 g) were subjected to fasting for at least 12 h prior to treatment. Then they were anesthetized with 1 g/kg of urethane administered intraperitoneally. An endotracheal tube with the 16 gauge needle front was inserted into the trachea. After the placement of catheter, a stethoscope was used to confirm the catheter was right in the trachea. Next, 100 μ L of PBS containing 2×10^5 CFU/mL MRSA was injected slowly to induce pneumonia. During the procedure, the rats were ventilated with a constant-volume respirator, and parameters were set as the following: inspired O₂ fraction of 1.0; peak airway pressures of 8–12 cm of H₂O and a 2 cm positive end expiratory pressure (PEEP); as high as 12 mL/kg tidal volume was given every 30 min to avoid potential positional pulmonary atelectasis; breathing frequency was controlled at 70 times/min. The respiratory rate was adjusted to maintain the pressure of CO₂ between 35 and 45 mmHg.

Pathological Analysis

A dose of 10 mg/kg weight of compound 13 was injected into the tail veins of tested rats intravenously. Time course profiles of histological changes in lungs of rats were assessed after intravenous injection followed by dissection after being euthanized. Sections from lungs were stained with hematoxylin and eosin (H&E) and observed under a light microscope. For pathological investigation of lungs of pneumonia suffering rats at different time points, sections were collected as above-mentioned and existence (indicated by the infiltration of neutrophils) and the extent of possible inflammatory response (indicated by the integrity of the alveolar structure and endothelium cilium) were recorded from five randomly picked sections.

Bronchoalveolar Lavage (BAL) Assay

The assay was conducted to estimate the situation of inflammatory reactions in lungs. Rats were sacrificed and exsanguinated via the femoral vessel under sterile condition. Then the thorax was opened, and the 18 gauge needle was introduced to the trachea at the cricothyroid membrane. Subsequently, 1 mL of sterilized PBS was administrated and extracted out for six cycles to promote the elution of inflammatory cells. Upon treatment, each specimen was homogenized. The suspension was subjected to enzyme linked immunosorbent assay (Elisa, R&D), and the level of tumor necrosis factor α (TNF- α) cytokine was investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MRSE	methicillin-resistant <i>Staphylococcus epidermidis</i>
<i>E. coli</i>	<i>Escherichia coli</i>
HDP	host-defense peptide
MIC	minimum inhibitory concentration
DAPI	4', 6-diamidino-2-phenylindole
PI	propidium iodide
CTC	2-chlorotrityl chloride
DIPEA	<i>N,N</i> -diisopropylethylamine
DIC	<i>N,N</i> -diisopropylcarbodiimide
HOBt	hydroxybenzotriazole
EtOAc	ethyl acetate
hRBC	human red blood cell
TSB	tryptic soy broth
CFU	colony-forming unit
PEEP	positive end expiratory pressure
H&E	hematoxylin and eosin
BAL	bronchoalveolar lavage

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Figure 1.

Structures of PMX-30063 and LTX-109 and design of small antimicrobial agents based on the acylated reduced amide. R₁ represents cationic groups. R₂, R₃, and R₄ represent hydrophobic groups.

Figure 2.
General chemical structures of α -peptide and reduced amide pseudopeptide.

Figure 3.

General scheme for the preparation of small reduced dipeptide derivatives.

Figure 4.

Fluorescence micrographs of *S. aureus* that are treated or not treated with 25 $\mu\text{g/mL}$ of **13** or **14** for 2 h: (a1) control, no treatment, DAPI stained; (a2) control, no treatment, PI stained; (b1) treatment with **13**, DAPI stained; (b2) treatment with **13**, PI stained; (c1) treatment with **14**, DAPI stained; (c2) treatment with **14**, PI stained. Scale bar = 10 μm .

Figure 5.

Time-kill curves of **13** (a) and **14** (b) for *MRSA*. The killing activity was monitored for the first 2 h. The concentrations were $4 \times \text{MIC}$, $8 \times \text{MIC}$, and $16 \times \text{MIC}$, respectively.

Figure 6.
Drug-resistance study of **13** and **14** toward MRSA.

Figure 7. Biological activity of compound **13** on inhibition of biofilm by *A. baumannii* and *E. coli*.

Figure 8.

In vivo efficacy of compound **13** on a MRSA-induced ventilator-associated pneumonia bearing rat model: (a) pathological assay via H&E staining; (b) TNF- α cytokine level in the rats by ELISA assay.

Table 1

Structures of Small Antimicrobial Molecules and Their Antibacterial Activity^c

#	Structure	MIC (µg/mL)						Hemolysis (HC ₅₀ , µg/mL)		IC ₅₀ (µg/mL)	
		Gram –			Gram +						
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	MRSA	MRSE	VRE	HK-2	K562		
1		>50	>50	>50	25–50	25–50	25–50	NT ^a	NT	NT	
2		25–50	6–12	25–50	25–50	25–50	25–50	NT	NT	NT	
3		>50	>50	>50	>50	>50	>50	NT	NT	NT	
4		>50	>50	>50	>50	>50	>50	NT	NT	NT	
5		25	12.5	25	25	12.5	25	290	NC ^b	NC	
6		50	25	25	25	12.5	25	300	>100	>100	

#	Structure	MIC ($\mu\text{g/mL}$)						Hemolysis (HC_{50} , $\mu\text{g/mL}$)		IC ₅₀ ($\mu\text{g/mL}$)	
		Gram –			Gram +					HK-2	K562
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	MRSA	MRSE	VRE				
7		12.5	6.12–12.5	12.5	6.25	6.25	12.5	225	NC	NC	NC
8		6.25	25	12.5	6.25	6.25	6.25	180	NC	NC	NC
9		6.25	12.5	12.5	6.25	6.25	6.25	160	NC	NC	NC
10		6.25	12.5	6.25	6.25	3.12	6.25	200	NC	NC	NC
11		6.25	6.25	6.25	6.25	3.12	6.25	120	NC	NC	NC
12		3.12	6.25	6.25	3.12	3.12	6.25	210	NC	NC	NC
13		3.12	6.25	6.25	1.56	3.12	6.25	85	86.3 $\pm$ 3.0	83.2 $\pm$ 4.3	

#	Structure	MIC (µg/mL)						Hemolysis (HC ₅₀ , µg/mL)		IC ₅₀ (µg/mL)	
		Gram –			Gram +						
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	MRSA	MRSE	VRE				
14		6.25	6.25	>25	1.56	3.12	6.25	280	NC	NC	
15		6.25	6.25	>25	3.12	3.12	6.25	200	NC	NC	
16		6.25	6.25	>25	3.12	3.12	6.25	250	NC	NC	
17	Daptomycin	>50	>50	>50	0.5	0.5	1				
18	Colistin	0.5	1	0.5	>50	>50	>50				

^aNT: not tested because compounds are not active.

^bNC: no cytotoxicity toward HK-2 and K562 cells at 25 $\mu\text{g/mL}$.

^cThe microbial organisms used in the study were *K. pneumoniae* (ATCC 13383), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), methicillin-resistant *S. epidermidis* (MRSE) (RP62A), methicillin-resistant *S. aureus* (MRSA) (ATCC 33591), and vancomycin-resistant *E. faecalis* (ATCC 700802). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits microbial growth after 20 h.^{24,37} HC₅₀ is the concentration causing 50% hemolysis. Hemolysis activity was not measured for peptidomimetics that did not exhibit antimicrobial activity. Daptomycin⁴¹ and colistin,⁴² which are last-resort antibiotics and active against either Gram-positive or Gram-negative bacteria, were included for comparison.