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High Cell Selectivity and Low-Level Antibacterial Resistance of Designed Amphiphilic Peptide G(IIKK)$_3$I-NH$_2$

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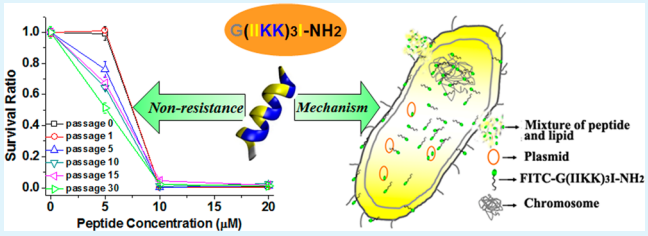
Supporting Information

ABSTRACT: On the basis of cell cultures involving bacterial strains (*Escherichia coli* 5α and *Bacillus subtilis* 168) and a mammalian cell line (NIH 3T3), the potent antibacterial activity and distinct selectivity from designed amphiphilic peptides G(IIKK)$_n$I-NH$_2$ (n = 2−4) have been demonstrated. This work extends these studies to multidrug resistant pathogens (ESBL-producing *E. coli*) and primary human cells (HDFa), followed by the in vivo mouse model investigation of ESBL-producing bacterial infection. G-(IIKK)$_3$I-NH$_2$ exhibits high antibacterial activity against the pathogenic strain both in vitro and in vivo while displaying low toxicity toward the primary cells and the mice. Peptide molecules can kill bacteria by selectively interacting with bacterial membranes, causing structural disruptions. Furthermore, multidrug resistant ESBL-producing bacteria do not develop resistance after multiple treatments with G(IIKK)$_3$I-NH$_2$. The high cellular selectivity, low toxicity toward mammalian hosts and noninducing bacterial resistance indicate great potential for developing the peptides as anti-infection agents.



KEYWORDS: *antimicrobial peptide, helical peptide, cell selectivity, ESBL-producing E. coli infection, membrane disruption, drug resistance*

INTRODUCTION

Increasing bacterial resistance to traditional antibiotics demands the development of novel therapeutic agents with different mechanisms of action.[1] Antimicrobial peptides (AMPs) have attracted attention because they not only act as direct antimicrobial agents, but also indirectly modulate the immune system against bacterial infection.[2−4] As antibiotics, most AMPs kill pathogenic bacteria via physical permeation and disruption of the membranes of the targeted cells whereas conventional antibiotics function by primarily interacting with specific receptors. It would appear to be a formidable task for bacteria to change the cell membrane structures and acquire antibiotic resistance. Following their early identification in insects by Boman,[5] more than 1000 native AMPs have been isolated and characterized from prokaryotes and eukaryotes.[6]

In spite of the unique advantages of natural AMPs, there are barriers to their development into therapeutic agents for treating bacterial infections, including high cost of production, poor proteolytic stability, potential toxicity to host cells and even unknown toxicology profiles when administered systemically.[7−9] To address these critical issues, many variants and mimics retaining the basic hallmarks of natural AMPs (e.g., cationic charge and amphiphilic structure) have been extensively explored, with some of them being in phase III clinical trials, including MSI-78, M-226 and hLF1-11 that are the analogues or derivatives of magainin-2, indolicidin and human lactoferricin, respectively.[7,10,11] In addition, computer-assisted peptide design strategies, combined with continuing advances in peptide synthesis, open up mechanistic studies aimed at resolving the inherent drawbacks of natural AMPs and bringing peptide antibiotics into the main stream of biomedical research.[7,12] For example, based on cecropins from insects, Loose et al. have optimized two potent sequences D28 (FLGVVFKLASKVFPAVFGKV) and D51 (FLFRVASKVFPA-LIGKFKKK) against *Bacillus anthracis* and *Staphylococcus aureus* by using a linguistic model.[12] However, the sequences that are too close to those of natural human AMPs can induce the inevitable compromise of human natural defense.[13]

Recently, we have designed a series of short helical peptides containing simple repeated IIKK sequences (G(IIKK)$_n$I-NH$_2$, n = 2−4).[14] These small and structurally simple peptides are cationic at the physiological pH of 7.4 and transform from a random coil to amphipathic α-helix when interacting with negatively charged membranes, following the structural features

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of most natural helical AMPs.[15] The peptide G(IIKK)$_3$I-NH$_2$ showed a potent antimicrobial ability comparable to the currently used antibiotic ampicillin against two type strains (*Escherichia coli* 5α and *Bacillus subtilis* 168) while displaying remarkably low hemolytic activity and high selectivity against bacteria when cocultured with NIH 3T3 fibroblast cells. The high cell selectivity of the peptide is ascribed to the combined use of these three factors: the tuning of the propensity of the α-helical conformation via the substitution of leucine residues by isoleucine, the perturbation of the hydrophobic face of the helix by the insertion of a positively charged lysine residue, and the optimization of the length of the peptide chain. In addition, the N-terminal glycine (G) capping and C-terminal amidation of AMPs were believed to favor their resistance to peptidases and stabilize α-helical structures.[16,17] These favorable properties thus allow us to make a systematic study of G(IIKK)$_3$I-NH$_2$ performance in systems that are closer to clinical use.

Bearing in mind clinical development of AMPs, we here extended the investigation utilizing primary cells (adult human dermal fibroblast cells, HDFa) rather than the cell line mouse NIH3T3 fibroblast as the model host cells and assessed the cell selectivity of G(IIKK)$_3$I-NH$_3$ in a coculture system containing both bacteria and HDFa cells. Because extended spectrum β-lactamase (ESBL)-producing organisms have been a major cause of human pathogen infections throughout the world due to their high level resistance to many currently used antibiotics,[18,19] we then examined the antibacterial ability of the peptide against ESBL-producing *E. coli* and in a mouse model with this bacterial infection. Finally, we assessed whether the process induced antimicrobial resistance in the treated microbes. In addition, the molecular mechanisms responsible for its antibacterial activity have also been investigated.

EXPERIMETNAL SECTION

1. Chemical Reagents. Rink amide MBHA resin, Fmoc protected amino acids and other chemical reagents and solvents used for peptide synthesis were obtained from GL Biochem Ltd. (Shanghai). Lipopolysaccharide (LPS, *E. coli* O111:B4) was purchased from Beyotime Institute of Biotechnology (China). 4′,6-Diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), propidium iodide (PI), dipalmitoyl phosphatidyl choline (DPPC), dipalmitoyl phosphatidyl glycerol (DPPG) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were used as received unless otherwise specified. Water used in all experiments was processed with a Millipore Milli-Q system (18.2 MΩ·cm).

2. Peptide Synthesis and Purification. G(IIKK)$_3$I-NH$_2$ was synthesized according to standard Fmoc based solid phase protocols on a commercial CEM Liberty microwave synthesizer and the detailed procedure has been described by us.[14,20] Note that Rink-amide MBHA resin (0.83 mM/g) was used to allow the C-terminus to be amidated and cleavage from the resin and side-chain deprotection were carried out by using a mixture of trifluoroacetic acid (TFA), water, and triisopropylsilane (TIS) (95:2.5:2.5, v/v) for 3 h at room temperature. To synthesize the N-terminal FITC labeled G(IIKK)$_3$I-NH$_2$ (FITC-G(IIKK)$_3$I-NH$_2$), a 6-aminohexanoic acid (Ahx) group was introduced between the last amino acid (G) and the fluorescein motif and such a step was postulated to favor the stabilization of the conjugate.[21] Its synthesis was also performed according to standard Fmoc based solid phase protocols, and the detailed procedures are included in our previous work.[14]

The crude peptide samples were repeatedly washed with cold ethyl ether at least eight times, then dissolved in an appropriate amount of Milli-Q water and finally freeze-dried in a lyophilizer. The resulting products were subjected to reverse-phase high performance liquid chromatography (RP-HPLC) and electrospray ionization-mass spectrometry (ESI-MS) characterizations, indicating their high purity (Figure S1 in the Supporting Information).

The purified peptides were dissolved in 0.01 M phosphate buffered saline (PBS, pH of 7.4) or water at a concentration of 2 mM, and then sterilized with a 0.22 μm filter. The stock solutions were subsequently frozen and kept at −20 °C prior to use.

3. Circular Dichroism (CD). CD spectra were measured on a Bio-Logic MOS 450 spectrometer using a quartz cell of 1 mm path length. Spectra were recorded from 190 to 250 nm at a scan speed of 50 nm/min. The peptide stock solution (2 mM) was diluted in water, 25 mM sodium dodecyl sulfate (SDS) and small unilamellar phospholipid vesicles (SUVs) aqueous solutions, respectively, with the final peptide concentration of 0.1 mM. Background spectra were obtained in the absence of peptide. Three scans were averaged to improve the signal-to-noise ratio. All measurements were carried out at room temperature and the CD signals are expressed as mean residual molar ellipticity.

SUVs composed of different phospholipids were prepared by ultrasonic treatment.[22] Typically, dry lipids (0.5 mg) were dissolved in 1 mL of chloroform in a small glass vessel and the solvent was removed by evaporation under vacuum overnight, resulting in a thin film on the glass vessel wall. Dried thin films were rehydrated in 1 mL of Tris−HCl buffer (10 mM, pH 7.4) by vortex mixing and then sonicated on ice with a titanium-tip ultrasonicator until the solution became clear.

4. Antibacterial Assay. The two type strains (*Escherichia coli* DH5α (*E. coli* DH5α) and *Bacillus subtilis* 168 (*B. subtilis* 168)) were obtained from the China Center of Industrial Culture Collection (Beijing). *E. coli* DH5α was grown in LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.0) and *B. subtilis* 168 was incubated in beef extract peptone medium (glucose 60 g/L, beef extract 10 g/L, peptone 10 g/L, yeast extract 10 g/L, NaCl 5 g/L, pH 7.2). ESBL-producing *E. coli* was a kind gift from Professor Xiaoxing Luo (Fourth Military Medical University, China) and was also grown in LB medium.[23]

The antibacterial activity of G(IIKK)$_3$I-NH$_2$ was measured by using a microplate autoreader (Molecular Devices, M^{2}e). Bacteria from the exponential growth phase were washed twice with PBS and resuspended in the media. Aliquots of 100 μL of bacteria (1×10^6 CFU/mL) were mixed with 100 μL of PBS containing the peptide in serial 2-fold dilutions in a sterile 96-well plate. The bacteria were incubated at 37 °C for 18 h. The absorbance at 600 nm (A600) was recorded. Survival rates were determined by the ratios of A600 readings of the bacterial cells treated with the peptide over those of the control bacterial cells without the peptide treatment. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the peptide that prevents visible growth of bacteria.[24]

To test the kinetics of bactericidal ability, aliquots of G(IIKK)$_3$I-NH$_2$ (100 μL, 10 μM) were added into the wells containing *B. subtilis* 168 bacteria (100 μL, 1×10^6 CFU/mL). Aliquots of 20 μL were removed at varying intervals (5, 10, 20, 30, 60 min) and plated on agar plate. The colonies were counted after incubation for 16−18 h at 37 °C.

5. Cell Selectivity of G(IIKK)$_3$I-NH$_2$ between Human Primary Cells and Bacteria. To assess the selective interaction of G(IIKK)$_3$I-NH$_2$ toward different cells, FITC-G(IIKK)$_3$I-NH$_2$ was introduced into a coculture system containing primary HDFa cells and *B. subtilis* 168. The normal HDFa cells (Lifeline Cell Technology, FC-0024) were preincubated in FibroLife S2 medium (Lifeline Cell Technology, LL-0011) supplemented with low serum growth supplement (2%). Upon reaching 80% confluence, the cells (1 mL, 1×10^5 cells/mL) were transferred into a 6-well plate containing sterile coverslips at the bottom. After overnight culture at 37 °C, the medium was replaced with 1 mL of *B. subtilis* 168 suspended in PBS with a density of 1×10^6 CFU/mL. Simultaneously, FITC-G(IIKK)$_3$I-NH$_2$ was added into the wells containing HDFa cells and *B. subtilis* 168 with a final peptide concentration of 20 μM. After incubation at 37 °C for 1 h, the bacteria in the suspension were isolated, followed by washing with PBS at least for three times to remove unbound FITC-G(IIKK)$_3$I-NH$_2$ molecules, and the adhesive HDFa cells were also washed with PBS. Finally, 10 μL of the bacterial suspension was dropped onto the coverslip covered

with HDFa cells and the distribution of FITC-G(IIKK)$_3$I-NH$_2$ was observed with a Leica DMI3000 fluorescence microscopy equipped with an oil immersion lens.

6. In Vivo Anti-infection with a Mouse Mode of Peritonitis. To determine the minimum concentration of bacteria that leads to mice death within 48 h of infection, the mice were assigned randomly into four treatment groups (five mice per group) and ESBL-producing *E. coli* bacteria were injected intraperitoneally at varying concentrations (with 0.5 mL injection volume). The lethal minimum concentration was determined to be about 5 × 10^9 CFU/mL (Table S1 in the Supporting Information).

After the mice were intraperitoneally injected with 0.5 mL of ESBL-producing *E. coli* (5 × 10^9 CFU/mL) for 1 h, G(IIKK)$_3$I-NH$_2$ dissolved in PBS was given by tail vein injection at a dose of 1.5 mg/kg each time. The peptide was injected into the mouse (body weight of about 20 g) twice per day (6 h interval each time during the daytime and the peptide injection was not given in the night) and the injection continued for 3 days. The control group was injected with PBS. The number of mice surviving at each group (five mice per group) was monitored for up to a period of 14 days after treatment. To assess the toxicity of the peptide to the mice major organs (liver and kidneys), blood samples were collected from the survival mice following the 14-day surveillance and the levels of alanine aminotransferase (ALT), asparate aminotransferase (AST), urea nitrogen and creatinine in blood were tested by ELISA. Simultaneously, the concentration of the bacteria in blood after the surveillance was also tested by the standard plate count method.

7. LPS Binding Assay. LPS was added into a 96-well plate with a concentration of 100 ng/well. The plate was incubated at 37 °C for 1 h, followed by washing with PBS. FITC-G(IIKK)$_3$I-NH$_2$ in PBS with or without $CaCl_2$ (1 mM) was then added into the LPS coated or uncoated wells. FITC in PBS with or without $CaCl_2$ (1 mM) was also added into the LPS coated or uncoated wells as the negative control. The final concentration of FITC-G(IIKK)$_3$I-NH$_2$ or FITC in each wells was 50 μM. Following incubation at 37 °C for 1 h, the wells were washed with PBS for at least three times and the fluorescence was recorded by the microplate autoreader (Molecular Devices, M^{2}e, excitation at 490 nm and emission at 530 nm).

8. Membrane Integrity. Bacterial membrane integrity was investigated by using a double-staining method with DAPI and FITC as dyes. After incubating with G(IIKK)$_3$I-NH$_2$ for 1 h at 37 °C at concentrations of 80 and 20 μM, respectively, *E. coli* DH5α and *B. subtilis* 168 bacteria (1 × 10^7 CFU/mL) were centrifuged at 3000 rpm for 5 min and washed with PBS, followed by treatment with 2.5% (v/v) glutaraldehyde in PBS for 1 h. For nuclear staining, 100 μL of DAPI solution (10 μg/mL) was introduced into the system. After incubation for 30 min at room temperature, bacteria were washed three times with PBS, and subsequently incubated with 100 μL FITC (6 μg/mL) for another 30 min in the dark. Finally, bacteria were washed, resuspended with PBS and observed using a Leica DMI3000 microscope equipped with an oil-immersion objective (100×). Negative control was performed with the same procedures but without the peptide treatment.

Bacterial morphology was tested by scanning electron microscopy (SEM). Briefly, 100 μL of *E. coli* DH5α and *B. subtilis* 168 (1 × 10^7 CFU/mL) were treated with 100 μL of G(IIKK)$_3$I-NH$_2$ at the concentrations of 80 and 20 μM, respectively, for 1 h at 37 °C. After centrifugation at 3000 rpm for 5 min and washing twice with PBS, the bacteria were fixed with 2.5% (v/v) glutaraldehyde in PBS. The samples were then observed with a JSM-840 instrument operated at 15 kV.

9. Location of G(IIKK)$_3$I-NH$_2$ in Bacteria. FITC-G(IIKK)$_3$I-NH$_2$ was added into the wells containing *E. coli* DH5α and *B. subtilis* 168 (1 × 10^7 CFU/mL) for 1 h at 37 °C at final concentrations of 80 and 20 μM, respectively. The samples were centrifuged and washed three times with PBS to remove unbound FITC-G(IIKK)$_3$I-NH$_2$ molecules. To assess cell viability, PI (5 μg/mL in PBS) was used to stain the treated and untreated bacteria with FITC-G(IIKK)$_3$I-NH$_2$. After the samples were fixed with 2.5% glutaraldehyde for 1 h at room temperature, 20 μL bacterial suspensions were immobilized on glass slides and observed with a Leica DMI3000 microscope.

10. Drug Resistant Simulation. To induce antimicrobial resistance, we first performed multiple treatments of bacteria with the peptide or ampicillin as reported previously.[23,25] Briefly, ESBL-producing *E. coli* bacteria from the exponential growth phase without treatments with peptide or ampicillin were regarded as the passage 0. After exposure to 3 μM G(IIKK)$_3$I-NH$_2$ or 135 μM ampicillin overnight at 37 °C, the bacteria were regarded as the first passage. Such a treatment was repeated for n times (n = 1−30 for the peptide, 30 days; n = 1−9 for ampicillin, 9 days) and the resulting bacteria were regarded as passage n bacteria.

Then, the antibacterial profiles of G(IIKK)$_3$I-NH$_2$ and ampicillin against the treated microbes were determined by using the microplate autoreader (Molecular Devices, M^{2}e). ESBL-producing *E. coli* bacteria of passage n in logarithmic growth phase were washed twice with PBS and resuspended in the media. Aliquots of 100 μL of bacteria (1 × 10^6 CFU/mL) were mixed with 100 μL of PBS containing the peptide or ampicillin in serial 2-fold dilutions in a sterile 96-well plate. The bacteria were incubated at 37 °C for 18 h. The absorbance at 600 nm (A600) was recorded. Survival rates were determined by the ratios of A600 readings of the bacteria treated with G(IIKK)$_3$I-NH$_2$ or ampicillin over those of the control bacteria of passage n.

RESULTS

1. Secondary Structure. CD study revealed that G(IIKK)$_3$I-NH$_2$ adopted a random coil conformation in water (Figure 1a, squares). But in a membrane-mimicking environment (generated by the addition of SDS[26]), it showed an α-helix rich structure, characterized by the occurrence of the two minimal mean residual molar ellipticities at 208 and 222 nm (Figure 1a, triangles). It has been proved that this serial of peptides can form a helical conformation in DPPG SUVs, while

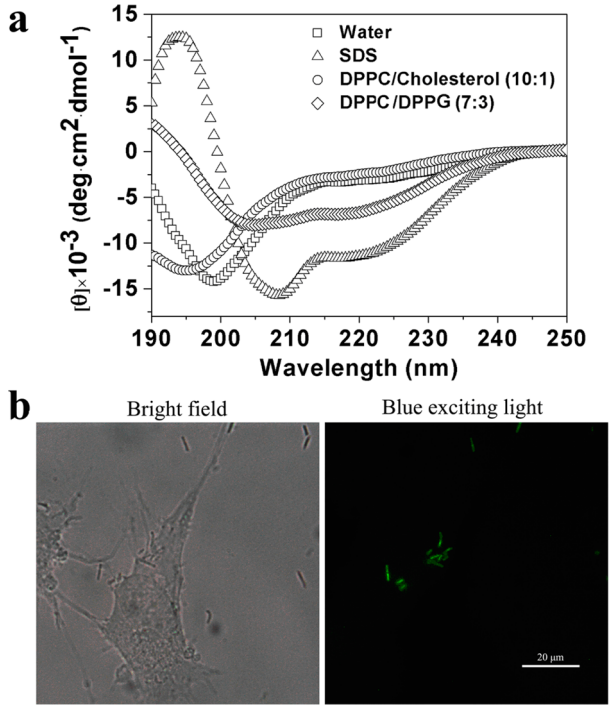

Figure 1. Secondary structures and cell selectivity of G(IIKK)$_3$I-NH$_2$. (a) CD spectra in water (open square), SDS (open triangle), DPPC/cholesterol (10:1, w/w, open circle) and DPPC/DPPG (7:3, w/w, open diamond) SUVs. The peptide concentration was fixed at 0.1 mM in different environments. (b) Cell selectivity in the coculture system containing *B. subtilis* 168 (rods) and HDFa primary cells (spindles).

retaining the random coil structure in DPPC SUVs.[14] Because there are considerable cholesterol molecules in mammalian cells membranes, we then applied SUVs composed of DPPC/cholesterol (10:1, w/w) as a model for normal mammalian cell membranes and of DPPC/DPPG (7:3, w/w) as a model for bacterial cytoplasmic membranes. When mixed with the mammalian membrane mimic DPPC/cholesterol SUVs (Figure 1a, circles), the peptide adopted the same random coil conformation as in the absence of the SUVs, indicating little interaction with the model mammalian membrane. In contrast, it adopted a distinct α-helical structure in the bacterial mimic DPPC/DPPG SUVs (Figure 1a, diamonds), showing strong affinity and interaction with the bacterial mimicking membrane. The difference shows the molecular basis of its good selectivity against different membranes.

The ratio of $[\theta]_{222}/[\theta]_{208}$ can be used as an additional criterion for evaluating the presence of α-helical coiled-coil structures.[27,28] For noninteracting α-helical coils, the ratio is typically less than 0.9, whereas for two-stranded coiled-coils, it is above 1.0. Based on the CD spectra of G(IIKK)$_3$I-NH$_2$ in SDS and DPPC/DPPG SUVs solutions (Figure 1a), the ratios are 0.71 and 0.87, respectively, suggesting that the peptide molecules might adopt a single-stranded helical structure in these bacteria mimicking membranes.

2. Antibacterial Activity, Cell Selectivity and in Vivo Anti-infection in a Mouse Model. The antibacterial activity of G(IIKK)$_3$I-NH$_2$ was first demonstrated using two type strains, *E. coli* DH5α and *B. subtilis* 168, as representative Gram-negative (G^-) and Gram-positive (G^+) bacteria. As shown in Figure 2a, little bacterial growth was observed at peptide concentrations of around 10 μM, suggesting that its MIC values against the two kinds of bacteria are close to 10 μM, and the peptide was more active against *B. subtilis* 168 than *E. coli* DH5α, consistent with our previous results.[14] Such potent bactericidal activity is comparable to those of currently used antibiotics such as ampicillin or a representative natural AMP (melittin) but clearly higher than that of magainin-2, another representative natural AMP.[29−31]

We then examined the bactericidal activity of G(IIKK)$_3$I-NH$_2$ against *B. subtilis* 168 with time at a concentration of 5 μM. It is very interesting that all *B. subtilis* 168 bacteria would lose their vitality within a period as short as 5 min (Figure 2a, inset). Such a rapid bactericidal ability suggests that the peptide may kill bacteria predominantly through physicochemical mechanisms on the cell surface.[4,32]

In spite of their potent antibacterial ability, natural AMPs such as melittin often have high hemolytic activity.[30,33] In contrast, our designed peptides have little toxicity to mammalian cells such as human red blood cells and other host cells.[14,34] The cell selectivity was then confirmed in this work through a coculture system containing both *B. subtilis* 168 and HDFa primary cells. The peptide was labeled with the green FITC tagging. As shown in Figure 1b, the green fluorescence from FITC was concentrated in the rod-shaped *B. subtilis* 168 bacteria rather than the adherent and spindle HDFa cells, indicating excellent cell selectivity of the peptide. It was also found during the coculturing that the binding of the peptide to the bacteria could be visualized within the first 5 min or so upon peptide addition, showing its rapid recognition to the bacteria.

Importantly, the peptide also showed potent antibacterial activity against ESBL-producing *E. coli* in vitro and there was no bacterial growth at a peptide concentration of 10 μM (Figure 2b), which is close to its effect against *E. coli* DH5α. The result thus confirms that the peptide is also effective against the infective and drug resistant strain and inhibiting its growth at a clinically practical dosage.

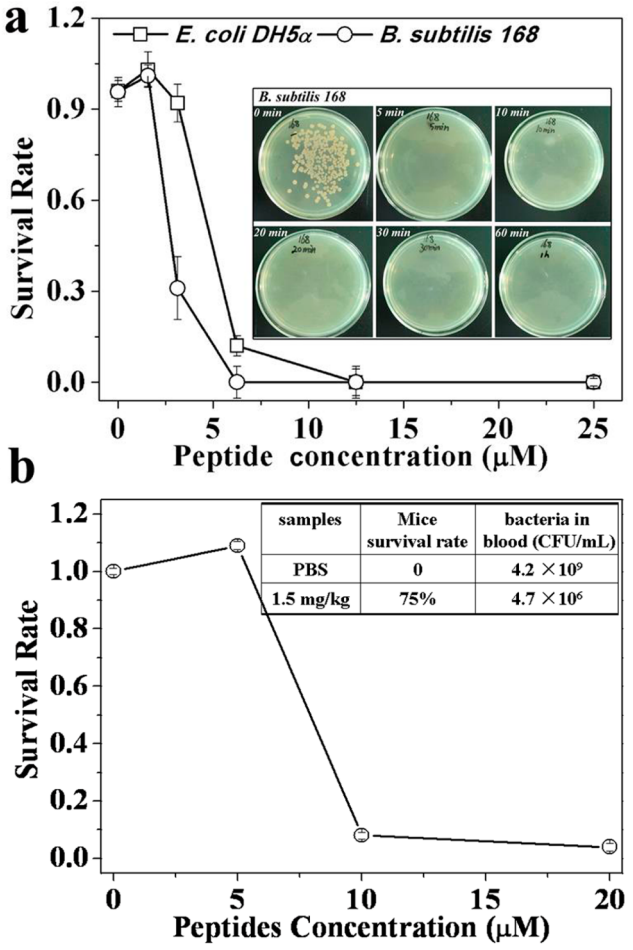

Figure 2. Antibacterial activity of G(IIKK)$_3$I-NH$_2$. (a) In vitro bactericidal curves for two type strains *E. coli* DH5α and *B. subtilis* 168 with respect to peptide concentrations. The inset shows the time dependent killing profile for *B. subtilis* 168 at a peptide concentration of 5 μM. (b) In vitro bactericidal curve for pathogenic ESBL-producing *E. coli*. The inset shows in vivo anti-infection activity of G(IIKK)$_3$I-NH$_2$ in the model mice infected with ESBL-producing *E. coli* at a lethal concentration of 5×10^9 CFU/mL, with survival rates and bacterial concentrations in blood being given. Note that the bacterial concentration in blood was determined after 48 h for the control group and that was determined following the 14 day surveillance for the peptide treated group.

For the mouse model, the minimum lethal concentration of ESBL-producing *E. coli*, leading to mice death within 48 h after intraperitoneal injection, was determined to be about 5×10^9 CFU/mL (with 0.5 mL injection volume, Table S1, Supporting Information). After the mice were infected for 1 h at this lethal concentration, the peptide was injected by tail vein at a dose of 1.5 mg/kg each time (twice per day, 3 days). For the control treated with PBS, there was no survival of the infected mice within 48 h after infection. For the treated mice, the survival rate was significantly increased to 75% at the end of 14 day surveillance (Figure 2b, inset). Furthermore, the concentration of ESBL-producing *E. coli* in the blood of survival mice after the 14 day surveillance was reduced significantly from 5×10^9 to 4.7×10^6 CFU/mL, i.e., by a factor of around 1000 (Figure 2b,

inset). Note that when the bacterial concentration in the mouse blood was below 6×10^7 CFU/mL, there was little effect on the mouse survival (Table S1, Supporting Information). These results indicate that the treatment of infected mice with G(IIKK)$_3$I-NH$_2$ can effectively inhibit bacterial growth and alleviate the degree of infection, thus increasing the survival rate significantly. The optimal dose and injection mode of the peptide are under investigation.

The levels of ALT, AST, creatinine and urea nitrogen in blood reflect liver and kidney functions.[35] We compared these parameters of the normal mice without infection and the infected ones receiving the peptide treatment (1.5 mg/kg). As shown in Figure S2 in the Supporting Information, the peptide treatment did not increase the levels of these key parameters, suggesting little toxicity of the peptide to the liver and kidney. Additionally, all the survival mice treated with the peptide were in good condition, without observable body weight loss.

3. Antimicrobial Mechanisms. *3.1. Interaction between G(IIKK)$_3$I-NH$_2$ and LPS.* There are several types of anionic molecules such as LPS and lipoteichoic acids (LTA) on the outer envelope of bacteria. Driven by the electrostatic attraction, cationic AMPs are likely to bind to them before interacting with the cytoplasmic membrane. We therefore assessed the binding of G(IIKK)$_3$I-NH$_2$ to LPS layers. First, we coated LPS molecules on the well bottom of a 96-well plate, and then added FITC-G(IIKK)$_3$I-NH$_2$ or FITC into the coated and uncoated wells and recorded the fluorescence intensity after washing with PBS. Almost no or only weak fluorescence signals were observed in either the FITC only addition wells or the LPS-uncoated wells (Figure 3a, histograms 1−6). However, the fluorescence was significantly enhanced in the LPS coated wells after incubation with FITC-G(IIKK)$_3$I-NH$_2$, suggesting that the peptide is able to interact effectively with LPS (Figure 3a, histogram 7).

Metal cations (primarily Ca^{2+} and Mg^{2+}) are well-known to interact with LPS within the cell envelope,[36] and as a result, a competition between them and cationic peptides was expected when interacting with LPS. However, the addition of Ca^{2+} ($CaCl_2$, 1 mM) did not inhibit the binding of G(IIKK)$_3$I-NH$_2$ to LPS but instead, it significantly enhanced their interaction (Figure 3a, histogram 8). Figure 3b shows that in the membrane mimicking SDS solution, G(IIKK)$_3$I-NH$_2$ formed α-helical structures irrespective of the presence or absence of $CaCl_2$, but the negative minima at 208 and 222 nm were both enhanced in the presence of $CaCl_2$. The result indicates that the addition of Ca^{2+} can promote the formation of the α-helical conformation of G(IIKK)$_3$I-NH$_2$, thus possibly favoring its interaction with LPS.

3.2. Membrane Permeabilization. Following binding with the outer leaflet of the bacterial membrane, G(IIKK)$_3$I-NH$_2$ molecules could aggregate on the membrane surface and disrupt the inner leaflet of the lipid bilayer, resulting in the internalization into cytoplasm. We then tested the integrity of the whole bacterial membrane via fluorescent-staining using DAPI and FITC as probes. No green fluorescence was detectable for the untreated bacterial samples (negative control) because of the intact cell membrane (Figure 4a). However, green fluorescence was noted in the bacteria after interaction with G(IIKK)$_3$I-NH$_2$ (Figure 4a), indicating that the cell membrane was disrupted by the peptide and the large fluorescent molecules subsequently accumulated inside the cytoplasm. All bacterial samples with DAPI staining gave positive results because DAPI inserts into the double strands of

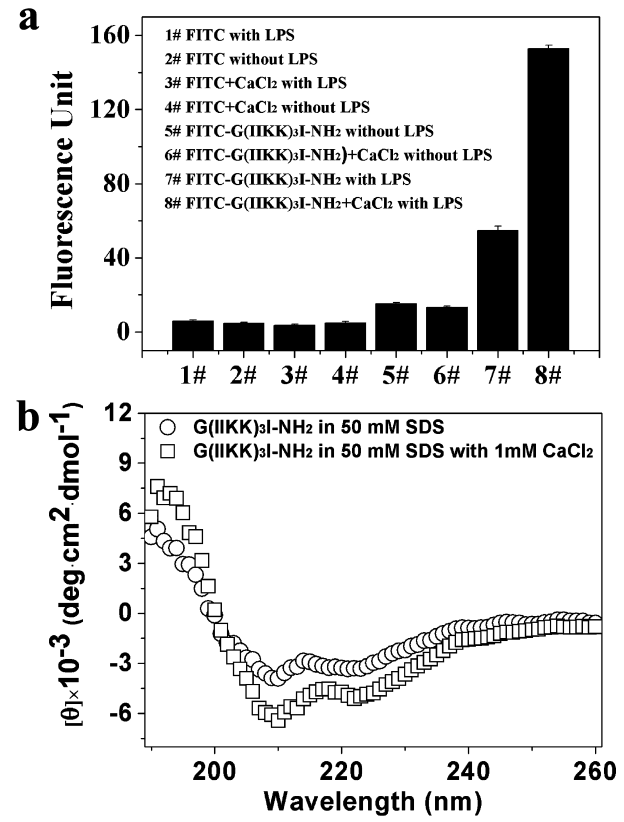

Figure 3. Interaction of LPS with G(IIKK)$_3$I-NH$_2$. (a) Fluorescence intensity of the LPS-coated wells after the incubation with FITC-G(IIKK)$_3$I-NH$_2$ or FITC in the presence or absence of $CaCl_2$. The uncoated wells served as the control. (b) CD spectra of G(IIKK)$_3$I-NH$_2$ in SDS solutions in the presence or absence of $CaCl_2$.

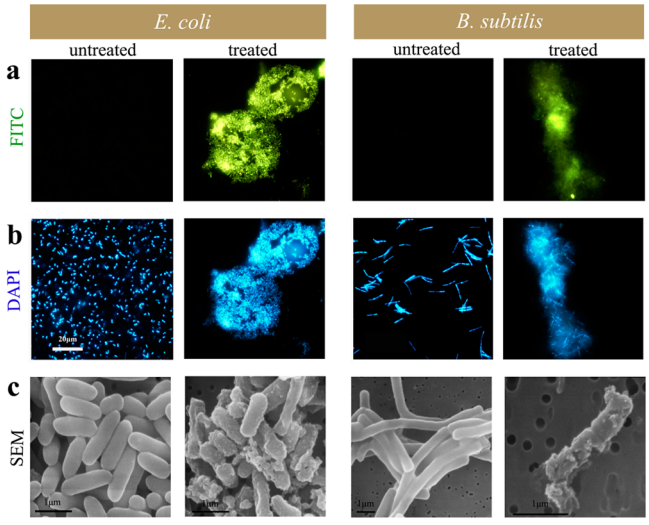

Figure 4. Plasma membrane integrity of *E. coli* DH5α and *B. subtilis* 168 before and after the peptide treatment. (a) FITC staining. (b) DAPI staining. (c) SEM images. As for the peptide treatment, *E. coli* DH5α and *B. subtilis* 168 bacteria were subjected to incubation with 80 and 20 μM G(IIKK)$_3$I-NH$_2$, respectively, for 1 h at 37 °C.

DNA molecules irrespective of the membrane integrity (Figure 4b). We also noticed that there are many large bacterial clusters for the G(IIKK)$_3$I-NH$_2$ treated samples, which may result from membrane fragments or leaked cytoplasm contents due to the membrane permeabilization (Figure 4a,b).

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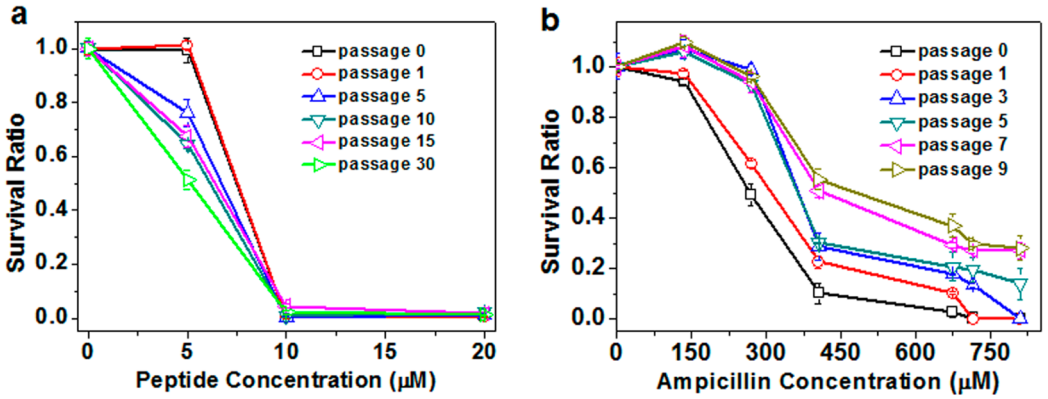

Figure 5. Development of bacterial resistance. (a) In vitro antibacterial profiles of $G(IIKK)_3I\text{-}NH_2$ against ESBL-producing *E. coli* bacteria after multiple treatments (n = 1−30) with the peptide at a concentration of 3 μM. (b) In vitro antibacterial profiles of ampicillin against ESBL-producing *E. coli* bacteria after multiple treatments (n = 1−9) with ampicillin at a concentration of 135 μM.

SEM experiments were performed under the same conditions as the fluorescence assay. As can be seen from Figure 4c, the untreated bacteria had a normal, smooth and intact surface. In contrast, different cell morphologies occurred after incubation with $G(IIKK)_3I\text{-}NH_2$. The majority of the bacteria showed surface roughening and blebbing and some cellular debris could be observed, which probably arose from cell lysis. In addition, some bacteria had large surface defects following treatment with the peptide. For individual cells, the damage was not uniformly distributed over the entire membrane areas.

After membrane disruption, the peptide is most likely to translocate into the cytoplasm. After treatment of *E. coli* and *B. subtilis* with FITC-$G(IIKK)_3I\text{-}NH_2$ for 1 h at higher concentrations, its green fluorescent signals markedly accumulated inside the *E. coli* and *B. subtilis* bacteria whereas there was no green fluorescence with the negative control bacteria (Figure S3 in the Supporting Information), suggesting internalization of the peptide after membrane permeabilization. In addition, FITC-$G(IIKK)_3I\text{-}NH_2$ was also found to be colocalized with PI which only penetrates into the damaged bacterial membrane and interacts with DNA and RNA in bacteria (Figure S3, Supporting Information), suggesting that the peptide is likely to interact with intracellular DNA and RNA to interfere the bacterial growth, but more information remained to be identified in the future.

4. Drug Resistance. Due to the membrane disruption mechanism, it should be rather difficult for bacteria to develop resistance to antimicrobial peptides. To test this point, we compared the antimicrobial activity of ampicillin and $G(IIKK)_3I\text{-}NH_2$ against ESBL-producing *E. coli* bacteria after their multiple treatments with the antibiotics at doses below the lethal concentrations. Due to the ability of hydrolyzing β-lactam antibiotics, ESBL-producing *E. coli* bacteria showed insensitivity toward ampicillin, with the MIC being around 650 μM (Figure 5b), significantly greater than those against *E. coli* DH5α and *B. subtilis* 168 (around 11 and 5 μM, respectively).[29] Furthermore, multiple exposures of the bacteria toward ampicillin below its MIC induced obvious resistance, with its inhibitory activity being significantly compromised with treatment times (Figure 5b, n = 1−9). In contrast, $G(IIKK)_3I\text{-}NH_2$ showed potent ability against the resistant bacteria, comparable to that against *E. coli* DH5α, and importantly, there was little alteration in its antimicrobial activity after the resistant bacteria were subjected to multiple treatments (n = 30) with the peptide (Figure 5a), suggesting no acquisition of resistance for the bacteria against the antimicrobial peptide.

DISCUSSION

Epidemics caused by ESBL-producing bacteria have been reported in many countries and have caused serious consequences for infection control worldwide. In China, ESBL-producing strains have become main pathogens of hospital infections besides methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE).[37] This is because these strains readily produce resistance to β-lactam antibiotics via hydrolysis, including ampicillin and the expanded-spectrum cephalosporins (e.g., third-generation ceftazidime, cefoperazone, cefotaxime and ceftriaxone) after multiple treatments with these antibiotics at relative low concentrations.[19,23] It is thus highly desired to develop novel antibiotics with a distinct mode of action. AMPs might be the most feasible candidates to replace conventional antibiotics, due to their targeting the whole cell membranes rather than specific receptors, which disfavors the development of resistance. Amphiphilic helical AMPs are often more active than peptides with less-defined secondary structures, thus representing a particularly successful development of the structural arrangement of native AMPs.[17,38]

The designed peptide $G(IIKK)_3I\text{-}NH_2$ adopted the non-ordered structure in water or when in contact with the zwitterionic (neutral) membrane. However, it transformed to the α-helical structure in the bacterial membrane-mimicking environments, even though isoleucine has a strong tendency to promote β-sheets.[39] The helical structure provides the molecule with an amphiphilic conformation in which the positively charged lysine residues are aligned along one side and the hydrophobic isoleucine residues on the other side. Such a facially amphiphilic conformation, together with its cationicity and hydrophobicity, would promote its interactions with bacterial outer and cytoplasmic membranes and subsequently cause damages.

As a result, the peptide displayed high toxicity against both G^+ and G^- bacteria (Figure 2a), comparable to synthetic antibiotic ampicillin (its MIC values around 11 and 5.5 μM against *E. coli* and *B. subtilis*, respectively[29]) and natural antimicrobial peptide melittin (its MIC values of around 4 and 2 μM against *E. coli* and *B. subtilis*, respectively[30]). However, melittin has low cell selectivity, causing 75% lysis of erythrocytes at concentrations around 0.6 μM.[30,33] In contrast,

$G(IIKK)_3I\text{-}NH_2$ showed low toxicity to mammalian cells such as NIH 3T3 cell line, human red blood cells and HDF primary cells, indicating it has high cell selectivity. Note that the typical nonhemolytic antimicrobial peptide, magainin-2, was active on both G^+ and G^- bacteria with its MIC values of 30−100 μM,[31,40] which were significantly higher than those of $G(IIKK)_3I\text{-}NH_2$. Note that the MIC values were determined by the same method, i.e., broth dilution.

Furthermore, the peptide also showed similar bactericidal activity against ESBL-producing *E. coli* bacteria (Figures 2b and 5a), which are currently regarded as a major threat to public health due to their multiple drug resistance (MDR). In contrast, ampicillin was insensitive to the resistant bacteria and multiple treatments readily induced the bacterial resistance, as shown in Figure 5b.

The high in vitro activity can translate to in vivo activity in model animals. When the mice were infected with ESBL-producing *E. coli* at a lethal concentration, the 3 day intravenous administration of the peptide significantly inhibited bacterial growth and saved 75% of the infected mice at a dose of 1.5 mg/kg each time. Additionally, the survived mice showed no noticeable weight loss and their key organs such as liver and kidneys were normal, suggesting again little toxicity of the peptide. Apart from the design strategies, i.e., the use of isoleucine, the perturbation of the hydrophobic face of the helix, and selection of the appropriate peptide chain length, the monomeric helical conformation of the peptide in the membrane also favors its lower toxicity.[41]

Similar to many natural helical AMPs, the peptide was found to kill bacteria primarily via membrane disruption. Such a mode of action is thought to disfavor the resistance acquirement of targeted bacterial cells. As a result, multiple treatments of ESBL-producing *E. coli* with the peptide did not induce antimicrobial resistance (Figure 5a). After membrane disruption, the peptide molecules accumulated within the cytoplasm and likely disturbed the intracellular targets.

Owing to these advantages, the peptide has great potential for practical applications. Its short and simple sequence not only favors its production on a large scale but also makes it easy to correlate its sequence and structure to bioactivity. Its ongoing and further characterizations will assist the development of peptide antibiotics with high potency against bacteria and little toxicity to host cells.

CONCLUSIONS

We have shown that the designed short helical $G(IIKK)_3I\text{-}NH_2$ could efficiently inhibit growth of multidrug resistant ESBL-producing *E. coli* bacteria in vitro and in vivo at clinically practical dosages while retaining low toxicity toward primary HDFa cells and the model mice. The peptide was found to kill bacteria primarily through membrane disruption, as a result of being cationic and taking amphiphilic α-helical conformation in the bacterial membrane-mimicking environments. As a mechanistic route of membrane damage, multiple treatments of ESBL-producing *E. coli* with the peptide did not induce antimicrobial resistance. Owing to these advantages, together with its short and simple sequence, the peptide holds great potential for practical applications as antibiotics agents.

ASSOCIATED CONTENT

Supporting Information

Figure S1, showing HPLC profiles and ESI-MS spectra of $G(IIKK)_3I\text{-}NH_2$ and FITC-$G(IIKK)_3I\text{-}NH_2$; Figure S2, showing the key physiological parameters in blood with the normal mice and the infected ones after the treatment of $G(IIKK)_3I\text{-}NH_2$; Figure S3, showing the location of $G(IIKK)_3I\text{-}NH_2$ within the treated bacteria; Table S1, showing the survival rate of the mice after 0.5 mL injection with ESBL-producing *E. coli* bacteria at different concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Nikaido, H. Multidrug Resistance in Bacteria. *Annu. Rev. Biochem.* **2009**, *78*, 119−146.

(2) Hancock, R. E. W.; Sahl, H. G. Antimicrobial and Host-Defense Peptides as New Anti-infective Therapeutic Strategies. *Nat. Biotechnol.* **2006**, *24*, 1551−1557.

(3) Yeaman, M. R.; Yount, N. Y. Mechanisms of Antimicrobial Peptide Action and Resistance. *Pharmacol. Rev.* **2003**, *55*, 27−55.

(4) Zasloff, M. Antimicrobial Peptides of Multicellular Organisms. *Nature* **2002**, *415*, 389−395.

(5) Steiner, H.; Hultmark, D.; Engström, Å.; Bennich, H.; Boman, H. G. Sequence and Specificity of Two Antibacterial Proteins Involved in Insect Immunity. *Nature* **1980**, *292*, 246−248.

(6) The Antimicrobial Peptide Database. http://aps.unmc.edu/ap/main.php (accessed March, 2014).

(7) Fjell, C. D.; Hiss, J. A.; Hancock, R. E. W.; Schneider, G. Designing Antimicrobial Peptides: Form Follows Function. *Nat. Rev. Drug Discovery* **2012**, *11*, 37−51.

(8) Peters, B. M.; Shirtliff, M. E.; Jabra-Rizk, M. A. Antimicrobial Peptides: Primeval Molecules or Future Drugs? *PLoS Pathog.* **2010**, *6*, e1001067.

(9) Yeung, A. T. Y.; Gellatly, S. L.; Hancock, R. E. W. Multifunctional Cationic Host Defence Peptides and Their Clinical Applications. *Cell. Mol. Life Sci.* **2011**, *68*, 2161−2176.

(10) Hancock, R. E. W.; Chapple, D. S. Peptide Antibiotics. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317−1323.

(11) van der Velden, W. J. F. M.; van Iersel, T. M. P.; Blijlevens, N. M. A. Donnelly, J. P. Safety and Tolerability of the Antimicrobial Peptide Human Lactoferrin I-II (hLFI-II). *BMC Med.* **2009**, *7*, doi:10.1186/1741-7015-7-44.

(12) Loose, C.; Jensen, K.; Rigoutsos, I.; Stephanopoulos, G. A Linguistic Model for the Rational Design of Antimicrobial Peptides. *Nature* **2006**, *443*, 867−869.

(13) Bell, G.; Gouyon, P. H. Arming the Enemy: The Evolution of Resistance to Self-proteins. *Microbiology* **2003**, *149*, 1367−1375.

(14) Hu, J.; Chen, C.; Zhang, S.; Xu, H.; Zhao, X.; Lu, J. R. Designed Antimicrobial and Antitumor Peptides with High Selectivity. *Biomacromolecules* **2011**, *12*, 3839−3843.

(15) Hancock, R. E. W. Cationic Peptides: Effectors in Innate Immunity and Novel Antimicrobials. *Lancet Infect. Dis.* **2001**, *1*, 156−164.
(16) Tossi, A.; Tarantino, C.; Romeo, D. Design of Synthetic Antimicrobial Peptides Based on Sequence Analogy and Amphipathicity. *Eur. J. Biochem.* **1997**, *250*, 549−558.
(17) Tossi, A.; Sandri, L.; Giangaspero, A. Amphipathic, α-Helical Antimicrobial Peptides. *Biopolymers* **2000**, *55*, 4−30.
(18) Rupp, M. E.; Fey, P. D. Extended Spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. *Drugs* **2003**, *63*, 353−365.
(19) Pitout, J. D. D.; Laupland, K. B. Extended-Spectrum β-Lactamase-producing Enterobacteriaceae: An Emerging Public-Health Concern. *Lancet Infect. Dis.* **2008**, *8*, 159−166.
(20) Chen, C.; Pan, F.; Zhang, S.; Hu, J.; Cao, M.; Wang, J.; Xu, H.; Zhao, X.; Lu, J. R. Antibacterial Activities of Short Designer Peptides: A Link Between Propensity for Nanostructuring and Capacity for Membrane Destabilization. *Biomacromolecules* **2010**, *11*, 402−411.
(21) Jullian, M.; Hernandez, A.; Maurras, A.; Puget, K.; Amblard, M.; Martinez, J.; Subra, G. N-Terminus FITC Labeling of Peptides on Solid Support: The Truth Behind the Spacer. *Tetrahedron Lett.* **2009**, *50*, 260−263.
(22) Ladokhin, A. S.; Fernández-Vidal, M.; White, S. H. CD Spectroscopy of Peptides and Proteins Bound to Large Unilamellar Vesicles. *J. Membr. Biol.* **2010**, *236*, 247−253.
(23) Bai, H.; You, Y.; Yan, H.; Meng, J.; Xue, X.; Hou, Z.; Zhou, Y.; Ma, X.; Sang, G.; Luo, X. Antisense Inhibition of Gene Expression and Growth in Gram-Negative Bacteria by Cell-Penetrating Peptide Conjugates of Peptide Nucleic Acids Targeted to *rpo*D Gene. *Biomaterials* **2012**, *33*, 659−667.
(24) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3*, 163−175.
(25) Wiradharma, N.; Khan, M.; Yong, L. K.; Hauser, C. A. E.; Seow, S. V.; Zhang, S. G.; Yang, Y. Y. The Effect of Thiol Functional Group Incorporation into Cationic Helical Peptides on Antimicrobial Activities and Spectra. *Biomaterials* **2011**, *32*, 9100−9108.
(26) Tulumello, D. V.; Deber, C. M. SDS Micelles as a Membrane-Mimetic Environment for Transmembrane Segments. *Biochemistry* **2009**, *48*, 12096−12103.
(27) Zhou, N. E.; Zhu, B. Y.; Kay, C. M.; Hodges, R. S. The Two-Stranded α-Helical Coiled-Coil is an Ideal Model for Studying Protein Stability and Subunit Interactions. *Biopolymers* **1992**, *32*, 419−426.
(28) Lau, S.; Taneja, A.; Hodges, R. Synthesis of a Model Protein of Defined Secondary and Quaternary Structure. Effect of Chain Length on the Stabilization and Formation of Two-Stranded α-Helical Coiled-Coils. *J. Biol. Chem.* **1984**, *259*, 13253−13261.
(29) Greenwood, D.; O'Grady, F. Differential Effects of Benzylpenicillin and Ampicillin on *Escherichia coli* and *Proteus mirabilis* in Conditions Simulating Those of the Urinary Bladder. *J. Infect. Dis.* **1970**, *122*, 465−471.
(30) Asthana, N.; Yadav, S. P.; Ghosh, J. K. Dissection of Antibacterial and Toxic Activity of Melittin: A Leucine Zipper Motif Plays a Crucial Role in Determining Its Hemolytic Activity But Antibacterial Activity. *J. Biol. Chem.* **2004**, *279*, 55042−55050.
(31) Wieprecht, T.; Dathe, M.; Beyermann, M.; Krause, E.; Maloy, W. L.; MacDonald, D. L.; Bienert, M. Peptide Hydrophobicity Controls the Activity and Selectivity of Magainin 2 Amide in Interaction with Membranes. *Biochemistry* **1997**, *36*, 6124−6132.
(32) Matsuzaki, K. Why and How are Peptide-Lipid Interactions Utilized for Self-Defense? Magainins and Tachyplesins as Archetypes. *Biochim. Biophys. Acta, Biomembr.* **1999**, *1462*, 1−10.
(33) Tosteson, M. T.; Holmes, S. J.; Razin, M.; Tosteson, D. C. Melittin Lysis of Red Cells. *J. Membr. Biol.* **1985**, *87*, 35−44.
(34) Chen, C.; Hu, J.; Zeng, P.; Pan, F.; Yaseen, M.; Xu, H.; Lu, J. R. Molecular Mechanisms of Anticancer Action and Cell Selectivity of Short α-Helical Peptides. *Biomaterials* **2014**, *35*, 1552−1561.
(35) Liu, L.; Xu, K.; Wang, H.; Tan, P. K.; Fan, W.; Venkatraman, S. S.; Li, L.; Yang, Y. Y. Self-Assembled Cationic Peptide Nanoparticles as an Efficient Antimicrobial Agent. *Nat. Nanotechnol.* **2009**, *4*, 457−463.
(36) Schindler, M.; Osborn, M. J. Interaction of Divalent Cations and Polymyxin B with Lipopolysaccharide. *Biochemistry* **1979**, *18*, 4425−4430.
(37) Yu, Y.; Ji, S.; Chen, Y.; Zhou, W.; Wei, Z.; Li, L.; Ma, Y. Resistance of Strains Producing Extended-Spectrum β-Lactamases and Genotype Distribution in China. *J. Infect.* **2007**, *54*, 53−57.
(38) Brogden, K. A. Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria? *Nat. Rev. Microbiol.* **2005**, *3*, 238−250.
(39) Minor, D. L. Jr; Kim, P. S. Measurement of the β-Sheet-forming Propensities of Amino Acids. *Nature* **1994**, *367*, 660−663.
(40) Zasloff, M.; Martint, B.; Chen, H. C. Antimicrobial Activity of Synthetic Magainin Peptides and Several Analogues. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 910−913.
(41) Bechinger, B.; Lohner, K. Detergent-like Actions of Linear Amphipathic Cationic Antimicrobial Peptides. *Biochim. Biophys. Acta, Biomembr.* **2006**, *1758*, 1529−1539.