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Original article

Tuning the antimicrobial pharmacophore to enable discovery of short lipopeptides with multiple modes of action

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ABSTRACT

Tryptophan and arginine rich antimicrobial peptides (AMPs) possess high potencies against both gram positive and gram negative bacteria, while lipopeptides represent another family of promising antimicrobial agents to combat invading pathogens. In the present study, we have synthesized a series of very short arginine, lysine and tryptophan containing lipopeptides and evaluated their antimicrobial activities against a panel of pathogenic microorganisms including *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The results showed that most of these peptides were effective against tested strains with MIC values ranging from 3.9 to 62.5 μg/mL. In addition to the small size, potent bactericidal activity, low to moderate hemolytic toxicity and membrane disruption ability, several peptides such as C10-RIKWWK and C10-RKWWK apparently retarded the migration of DNA on agarose gel in the DNA-binding assay, which implied the multiple modes of action in their bacteria-killing mechanism. These peptides revealed a promising therapeutic potential to develop as new antimicrobial agents.

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1. Introduction

The rapidly growing bacterial resistance to conventional antibiotics has become a worldwide problem spurring a strong need to develop new antimicrobial agents. To address this concern, antimicrobial peptides (AMPs) have emerged as an alternative strategy for the treatment of infectious diseases caused by resistant pathogens. These molecules are an ancient, evolutionarily conserved class of compounds that are widely distributed in mammals, invertebrates, plants and microorganisms, and provide the first line of innate and possibly adaptive immune defense against invading pathogens [1]. Their structures vary from 12 to 50 residues and differ in sequence and secondary structure but always fold into an amphipathic conformation upon interaction with biological membranes, and possess net positive charges which preferentially allow them to interact with negatively charged bacterial membranes [2]. AMPs have broad antimicrobial activities against bacteria, fungi, viruses and parasites, especially against those multiple antibiotic resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE), drug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. Although co-evolution with microbes over millions of years, AMPs are still highly effective and show low propensity for drug resistance development due to their unique modes of action compared with conventional antibiotics [3]. Despite these favorable features for a prospective drug, thus far, few AMPs have reached clinical utility owing to the issues with respect to the bioavailability, toxicity, stability and production cost. To improve their properties and make them more druggable, rational design is an attractive approach to addressing these issues. Among a large number of researches in this field, we are particularly interested in very short antimicrobial peptides composed of simple amino acids, which will not only reduce production costs, facilitate structural optimization, but also significantly reduce the potential to induce immunogenic response.

With regard to designing short antimicrobial peptides, we observe that many naturally occurring AMPs including lactoferrin, tachyplesin, defensin, indolicidin and protegrin [4–6] encompass tryptophan (W) and arginine (R) residues, which implies that these amino acids play a crucial role in AMPs. Not only are these residues abundant in naturally occurring AMPs, but also they are always presented in synthetic congeners [7]. It was reported that upon screening of a combinatorial library composed of millions of mixed hexapeptides, peptides rich in these two amino acids were proved to possess the highest antimicrobial activities [8]. In our previous

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work, by employing seven residues comprising only tryptophan (W), arginine (R) and phenylalanine (F), we generated several potent AMPs inhibiting the growth of a dental cavity causing bacterium of *Streptococcus mutans* with the basic structures of RWRWRWF and RRRWWWF [9]. In 2008, Haug et al. reported a potent small synthetic AMP and its homologues by covalently incorporating three bulky groups of t-butyl into the indole ring of W residue within a structural motif of R–W–R [10], which further proved the potential of R and W in the construction of ultra short AMPs.

Apart from classic host-defense cationic antimicrobial peptides (AMPs), it is well known that lipopeptides are another family of candidates to combat invading pathogens, some of which have shown potent antibacterial or antifungal properties thereby being brought into clinical or preclinical trials [11,12]. These lipopeptides consist of short linear or cyclic peptidyl head, linked to a fatty acid tail via ester or amide bond or both [13]. The detailed microbicidal mechanism of them is not fully elucidated; nevertheless, some evidences have shown that they may involve the disruption of bacterial membrane similar to that of many classical AMPs or act intracellularly [14]. Hence, the high potency and diverse structures of lipopeptides provide us another choice in construction of potent antimicrobials for the development of drugs against infectious diseases. On the basis of this progress, in the present study, we hypothesized that effective AMPs could be prepared by combining the advantages of lipopeptides with those of RW rich peptides. We then systematically designed and synthesized a series of short lipopeptides by conjugating a fatty acid lipid tail to the N-terminus of various RW containing peptidyl heads. These peptides were tested for their antimicrobial activities against a panel of pathogenic microorganisms including the gram-negative rod *Escherichia coli*, the gram-positive cocci *S. aureus* and yeast of *Candida albicans*. Their toxicity against human erythrocytes and mode of action were also discussed.

2. Materials and methods

All 9-fluorenylmethoxy carbonyl (Fmoc) L-amino acids, 2-(H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and rink amide 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-MHBA (MBHA) resin were purchased from GL Biochem Ltd. (Shanghai, China). Fatty acid of tetradecanoic acid (myristic acid) was obtained from Sigma–Aldrich (USA). All other fatty acids, coupling reagents and solvents were purchased from Aladdin Co. (China) with peptide synthesis grade.

2.1. Peptide synthesis

All peptides were synthesized by using standard 9-fluorenylmethoxy carbonyl (Fmoc) solid phase protocols on Rink Amide MHBA resin. The sequences were assembled on an ABI 433A peptide synthesizer with 0.1 mmol scale. Peptide elongation was effected by using standard HBTU/HOBt coupling chemistry in dimethylformamide (DMF) solvent with twofold molar excess of diisopropyl ethylamine (DIEA) in N-methyl-2-pyrrolidone (NMP) and eightfold excess Fmoc-protected amino acid or tenfold excess free fatty acid. Fatty acids were coupled using standard amino acid coupling conditions employed for all syntheses. All peptides were cleaved from the resin using reagent M, which contains 87.5% trifluoroacetic acid, 2.5% ethanedithiol, 5% thioanisole and 5% deionized water (3 h, room temperature), following standard work-up (crude product was precipitated in t-butyl methyl ether and washed twice with the same solvent). Peptide fluorescence labeling was proceeded as the same procedure as amino acid coupling by employing rhodamine as acid moiety while elongated the reaction time to 12 h.

The molecular weight of each peptide was confirmed by electrospray ionization mass spectrometry (ESI-MS, Waters). MS was performed using a Waters 3100 single-quadrupole mass spectrometer in the positive mode, specifically with a capillary voltage of −3 kV and cone voltage of −30 V. Nitrogen was used for both the cone gas (50 l h^{-1}) and desolvation gas (650 l h^{-1}), with the source and desolvation temperatures being held at 350 °C, respectively. The results were as detailed in Table 1. Peptide purity was analyzed by RP-HPLC using a Shimadazu 10A HPLC with C18, 250 × 4.6 mm column (Shimadzu, Japan), The HPLC listed in Table 1 is as follows: flow rate, 1 mL/min; mobile phase, solvent A: water (0.1% trifluoroacetic acid), B: acetonitrile (0.1% trifluoroacetic acid); gradient:15%–20% B (2 min), 20%–60% B (6 min), 60%–80% B (4 min), 80%–90% B (4 min). All lipopeptides were found to have a purity of 85–90%.

2.2. Strains and growth conditions

The following strains were used in this study: *S. aureus* ATCC 12600, *E. coli* ATCC25922 and *C. albicans* ATCC14053. All microbial strains were stored at −80 °C in Microbank vials and grown under aerobic conditions. Of the strains used in this study, *E. coli* ATCC25922 were grown at 37 °C in Luria–Bertani broth medium (LB), *S. aureus* ATCC 12600 was grown in Todd–Hewitt (TH) broth medium at 37 °C and *C. albicans* in YPD medium (1% yeast extract, 1.5% peptone, 2% dextrose) at 30 °C.

2.3. Minimum inhibitory concentration determinations (MIC)

MIC tests were carried out according to CLSI guidelines with minor modifications [9]. Briefly, overnight bacterial strains (1×10^8 CFU/mL) grown at 37 °C were diluted with media to a final bacterial concentration of 1×10^5 CFU/mL in all antibacterial assays. MICs were determined as the lowest concentration of peptide that inhibits bacterial growth when incubated in 50% strength of MH

Table 1
Name, sequence and physicochemical properties of designed peptides.[a]

Name	Sequence	Ch[b]	%Hp[b]	MH[b,c]	pI[b]	RT[d]	MW	
							Calc.	Obs.(M+H)
AMP-C14-1	KWWK	2	50	−0.1	10.8	12.44	856.1	856.7
AMP-C14-2	KIWWK	2	60	1.65	10.8	12.6	969.3	969.7
AMP-C14-3	RKWWK	3	40	−2.08	11.82	11.61	1012.3	1012.7
AMP-C14-4	RIKWWK	3	50	−0.28	11.82	11.1	1125.4	1126.4
AMP-C14-5	RWWR	2	50	−0.15	12.5	12.92	912.1	912.7
AMP-C14-6	KRIWWR	3	50	−0.3	12.51	11.42	1153.5	1154.4
AMP-C14-7	KIKRWWR	4	43	−1.67	12.53	10.84	1281.7	1282.8
AMP-C14-8	KIRWWR	3	50	−0.3	12.51	11.72	1153.5	1153.8
AMP-C14-9	RWR	2	33	−3.43	12.5	11.93	725.9	726.8
AMP-C14-10	KIKRWR	4	33	−3.56	12.53	10.52	1095.4	1095.7
AMP-C14-11	KKIRWR	4	33	−3.56	12.53	10.35	1095.4	1096.6
AMP-C14-12	KWK	2	33	−3.36	10.8	11.37	669.9	670.6
AMP-C14-13	KKWK	3	25	−5	11.1	10.54	798.1	798.8
AMP-C14-14	RIKWK	3	40	−2.28	12.53	11.04	939.2	939.8
AMP-C14-15	RWRW	2	50	−0.15	12.5	12.45	912.2	912.7
AMP-C14-16	KWKW	2	50	−0.1	10.8	12.09	856.1	856.7
AMP-C14-17	KKWW	2	50	−0.1	10.8	11.4	856.1	856.7

[a] The N-termini of peptides were conjugated with a C14 fatty acid, and the C-terminal were amidated.
[b] Charge (Ch) and PI and percentage of hydrophobicity (%Hp) were calculated based on amidated peptide alone without considering lipid portion by using online tool https://www.genscript.com/sslbin/site2/peptide_calculation.cgi.
[c] The value of MH (mean hydrophobicity) was calculated based on Kyte&Doolittle scale by using website at http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html#run.
[d] Retention time (min).

broth at 37 °C for 17–20 h in a stationary incubator, the antifungal susceptibility was evaluated from the turbidity visible in 50% strength of YPD broth after 20 h of incubation at 30 °C. Peptide concentrations varied from 31.2 mg/mL to 0.97 μg/mL in serial two-fold dilutions for bacteria, and from 62.5 to 1.98 μg/mL for *C. albicans*. All assays were performed parallelly in duplicate with four to six independent experiments. The susceptibility of the bacterial strains against neomycin, C16-KGGK [16] was used as an internal standard during MIC determinations. The results (MIC values in μg/mL) for neomycin were the following: *E. coli*, 3.2; *S. aureus*, 3.2; For peptide C16-KGGK: *E. coli*, 31.2; *S. aureus*, 7.8.

2.4. Hemolytic activity

The hemolytic activities of selected most potent peptides were determined in fresh human red blood cells (RBCs) according to reported procedure [13]. Briefly, fresh human erythrocytes were washed three times with an equal volume of PBS, and then resuspended in 10% (v/v) in PBS (10 mM phosphate buffer, pH 7.0, 150 mM NaCl). Equal volumes (100 μL) of the erythrocyte suspension were added to each well of a 96-well microtitre plate containing an equal volume of each peptide in PBS at concentrations of 62.5, 125 and 250 μg/mL. The mixtures were incubated for 1 h at 37 °C, and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant (50 μL each well in the 96-well microtitre plate) at 570 nm was then measured. PBS and 10% Triton X-100 were used as negative and positive hemolysis controls, respectively. The hemolysis percentage was calculated as follows:

$$\text{Haemolysis}\% = \left[\left(\text{Ab}_{\text{peptide}} - \text{Ab}_{\text{PBS}}\right) \Big/ \left(\text{Ab}_{\text{Triton}} - \text{Ab}_{\text{PBS}}\right)\right] \times 100$$

2.5. MTT assay

Human HeLa cells (TZM-bl) (Southern Medical University, China) were cultured in 96-well plates (Corning, USA) with DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco), 1% penicillin/streptomycin. The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere. TZM-b1 cell cultures (1×10^5 cells/mL, 100 μL in each well) were prepared in 96-well plates and incubated for 24 h. After incubation, the culture was removed and then 100 μL of the same medium containing various concentrations (15.6, 31.3, 62.5, 125.0, 250.0, and 500.0 μg/mL) of peptide were added into each well and further incubated at 37 °C for 48 h. After completion, the culture supernatants were removed and 100 μL of 0.5 mg/mL MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltet-razolium bromide) solution was added to each well. The plates were incubated for 4 h at 37 °C covered with aluminum film, then the MTT solution was removed and 150 μL DMSO was added to dissolve formazan crystals. The absorbance at 570 nm was recorded on a Microplate Reader (Tecan, Swiss). The percentage of viability was calculated as AT/AC × 100, where AT and AC refer to the absorbance of reacted and control cells, respectively.

2.6. Flow cytometry analysis of peptide toxicity

TZM-b1 cells were grown in high glucose DMEM plus 10% calf serum medium. Cells propagated at sub-confluency levels were washed with PBS, and detached with trypsin-EDTA (0.05% and 0.02% w/v, respectively) solution. The suspension of harvested cells was diluted with 10 volumes of growth medium, sedimented at 800 rpm for 5 min and re-suspended in high glucose DMEM plus 10% calf serum at a density of 2×10^5 cells/mL. AMP-C10-3 and AMP-C10-14 at 40 μg/mL were respectively added to the cells, and at the end of each time point (1 h and 2 h respectively), propidium iodide (PI, 10 μg/mL) was added to an aliquot of cells to determine the viability of cells. As a negative control, the number of cells in the presence of PI only was normalized as 100% viable cells.

Propidium iodide produces cytotoxic effects only after long exposure (>60 min) resulting in ca. 10% positive cells. To minimize this problem, PI was added to each sample just prior to sorting. The time points indicated in Fig. 3 reflect the time after addition of the peptides, not the addition of PI.

2.7. Determination of bactericidal kinetics

The short-term killing effect of peptides was determined by time-kill experiments [9]. In brief, *S. aureus* was diluted to 1×10^6 CFU/mL in MH medium, 40 μg/mL (10 excess of MIC) peptide was added to the cell suspension and incubated at 25 °C. At each time point (0, 60, 120 min), 10 μL of the cell suspension was removed, diluted in growth medium (1:50), and kept on ice prior to plating. CFU/mL was calculated after 24 h incubation at 37 °C under aerobic conditions.

2.8. PI uptake assay

Two equally charged peptides AMP-C10-14 (250 μg/mL) 20 μL and AMP-C10-3 (250 μg/mL) 20 μL were added into 70 μL of overnight culture of *E. coli* and *S. aureus* containing 10 μL propidium iodide (PI), respectively. The mixture was incubated at room temperature for 20 min, then washed with PBS twice and observed under the fluorescence microscope (Nikon, Japan). With the same procedure, by comparison, 16 μL of AMP-C10-3 (25 μg/mL) was also incubated with 74 μL of *E. coli* and *S. aureus*, respectively, and then went to observe the red fluorescence.

2.9. DNA binding assay

The DNA binding experiments were performed for peptides as described previously [6]. Briefly, 347 ng of the plasmid DNA (pHis1525) was mixed with two different concentrations (25 and 50 μg/mL respectively) of peptides in 20 μL of buffer (10 mM Tris–HCl, 1 mM EDTA buffer, pH 8.0). Reaction mixtures were incubated at room temperature for 30 min. Subsequently, native loading buffer was added, and a 20 μL aliquot was subjected to 1.0% agarose gel electrophoresis. The migration of DNA was detected by the fluorescence of ethidium bromide (Bio-Rad).

2.10. Statistical analysis

Data were statistically analyzed by one-way analysis of variance and Student's test. The graph was processed using GraphPad Prism software, version 5.0.

3. Results

3.1. Peptide design

It has been known that in spite of displaying a weak activity, R–W dipeptide accounts for a minimal motif or pharmacophore for antimicrobial activity which is always presented in the domain of naturally occurring antimicrobial peptides as well as in synthetic counterparts [9,10]. This is partially due to the existence of cation–π interactions imparted from aromatic tryptophan and positive charged residues such as arginine and lysine, which favors peptide membrane interactions [15]. The amino acid of tryptophan (W) resides at the membrane interface and disrupts the hydrophobic interactions of the lipid acyl chains within membrane lipid bilayer

[15]. In contrast, both arginine (R) and lysine (K), naturally occurring polar and positively charged residues at the physiological condition, are presented in almost all cationic antimicrobial peptides to represent the charged moiety. Although in some cases these two residues are interchangeable, we believe that owing to the intrinsic differences in pH value, ion strength and lipophilicity, they would show a different interaction and selectivity toward different cell membranes. Thus, the unique physicochemical properties of these amino acids make them suitable building blocks for the construction of antimicrobial agents. Taken together, various small R, K and W containing lipopeptids based on four predefined templates of RWR, KWK, RWWR and KWWK with a minimum charge of +2 were designed. Without considering the secondary structure, the length of sequences was limited within seven amino acids and elongated by adding residues of R, K, W and I to change their charge, aromaticity, and hydrophobicity. The distance between two lysines (or arginines) was also varied with the aim to enhance their potencies. To provide the lipophilicity, in our initial library, a linear C14 fatty acid of myristic acid was covalently conjugated to the N-terminus of each peptide, and the C termini of all these peptides were amidated to improve their proteolytic stability. The sequences and physicochemical properties of investigated peptides are listed in Table 1.

3.2. Antimicrobial activities of peptides

Peptides were tested for their antimicrobial activity against gram positive and gram negative bacteria of *S. aureus* and *E. coli*, and yeast of *C. albicans*. Table 2 shows that most of these peptides were more effective against *S. aureus* than *E. coli*, while not active against *C. albicans* in our test range. The antibacterial activity against *S. aureus* was virtually no significant differences between all sequence variations ranging from simple RW or KW containing sequences to the peptides mixed with R, K, W and I residues. Clearly, the antimicrobial activity is related to their physicochemical properties especially in the charge and hydrophobicity. A simple change in charge or hydrophobicity will affect their potencies, e.g. after inserting an isoleucine residue into peptide AMP-C14-1, the minimal inhibitory concentration (MIC) against *S. aureus* of nascent peptide AMP-C14-2 decreased up to four-fold.

The initial results inspired us to speculate that how the antimicrobial profile of this library would be if changing the length of lipid tail? We then synthesized a second library based on the preliminary results obtained from Table 2 by replacing the lipid tail of C14 with linear C10 and C18 fatty acid respectively. The antimicrobial activities summarized in Table 3 revealed that most peptides substituted with C10 fatty acid were comparatively more potent than the prototype of C14 homologues, whereas derivatives substituted with C18 fatty acid showed an opposite effect.

Interestingly, both C_n-RWWR and C_n-KWWK (n = 10, 14) displayed a potent antimicrobial activity. To gain more insights into the physicochemical properties and antimicrobial profile of these peptides, we then studied whether their potencies would be improved by varying the order of their sequences as RWRW, KWKW and KKWW. As shown in Tables 2 and 3, all these nascent peptides, especially C10 substituted congeners, exhibited broad and potent activities with MIC values as low as 3.9 μg/mL, indicating that the antimicrobial activities of these four residual peptides were mainly governed by their charge and lipophilicity, while less affected by the order of their residues.

3.3. Evaluation of hemolytic activity of selected potent peptides

A significant concern in the development of antimicrobial peptides as a clinically useful drug is their toxicity to host cells. The candidates should be able to discriminate between mammalian and bacterial cells. In the present study, the toxicity of selected peptides is evaluated by using human erythrocytes to check for their hemolytic activity. As shown in Fig. 1, the hemolytic effect was strongly related to the number and the position of W residue. The more W residues there are, the higher of lytic effect would be. A

Table 2
Minimal inhibitory concentration (MIC) of designed peptides.[a]

Name	Sequence	MIC (μg/mL)		
		S. aureus ATCC 12600	*E. coli* ATCC25922	*C. albicans* ATCC14053
AMP-C14-1	KWWK	3.9	7.8	15.6
AMP-C14-2	KIWWK	7.8–15.6	31.2	31.2
AMP-C14-3	RKWWK	7.8	15.6–31.2	>62.5
AMP-C14-4	RIKWWK	7.8–15.6	15.6–31.2	>62.5
AMP-C14-5	RWWR	7.8–15.6	15.6–31.2	>62.5
AMP-C14-6	KRIWWR	7.8	15.6–31.2	>62.5
AMP-C14-7	KIKRWWR	15.6	31.2	>62.5
AMP-C14-8	KIRWWR	15.6	31.2	>62.5
AMP-C14-9	RWR	15.6	15.6–31.2	>62.5
AMP-C14-10	KIKRWR	3.9	15.6–31.2	>62.5
AMP-C14-11	KKIRWR	3.9	7.8	>62.5
AMP-C14-12	KWK	7.8–15.6	7.8–15.6	>62.5
AMP-C14-13	KKWK	3.9	7.8–15.6	>62.5
AMP-C14-14	RIKWK	15.6	15.6–31.2	>62.5
AMP-C14-15	RWRW	15.6–31.2	15.6–31.2	>62.5
AMP-C14-16	KWKW	3.9	7.8–15.6	>62.5
AMP-C14-17	KKWW	7.8	15.6	15.6

All MIC determinations were repeated 4–6 times.
[a] Neomycin sulfate and C16-KGGK [16] were used as positive control.

Table 3
Minimal inhibitory concentration (MIC) of designed peptides.

Name	Sequence	MIC (μg/mL)		
		S. aureus	*E. coli*	*C. albicans*
AMP-C10-1	KWWK	7.8–15.6	15.6–31.2	31.2
AMP-C10-2	KIWWK	3.9–7.8	7.8–15.6	15.6–31.2
AMP-C10-3	RKWWK	3.9	3.9–7.8	15.6–31.2
AMP-C10-4	RIKWWK	3.9	7.8–15.6	>62.5
AMP-C10-5	RWWR	7.8–15.6	15.6–31.2	15.6–31.2
AMP-C10-6	KRIWWR	3.9–7.8	7.8	>62.5
AMP-C10-7	KIKRWWR	3.9	7.8	>62.5
AMP-C10-8	KIRWWR	3.9	15.6	>62.5
AMP-C10-9	RWR	7.8	15.6	>62.5
AMP-C10-10	KIKRWR	15.6	31.2	>62.5
AMP-C10-11	KKIRWR	7.8	7.8–15.6	>62.5
AMP-C10-12	KWK	31.2	>31.2	>62.5
AMP-C10-13	KKWK	31.2	>31.2	>62.5
AMP-C10-14	RIKWK	7.8	15.6–31.2	>62.5
AMP-C10-15	RWRW	7.8–15.6	15.6–31.2	>62.5
AMP-C10-16	KWKW	7.8–15.6	7.8–15.6	31.2
AMP-C10-17	KKWW	3.9–7.8	7.8	31.2
AMP-C18-1	KWWK	>31.2	>31.2	>62.5
AMP-C18-2	KIWWK	>31.2	>31.2	>62.5
AMP-C18-3	RKWWK	31.2	125	>62.5
AMP-C18-4	RIKWWK	31.2	>31.2	>62.5
AMP-C18-5	RWWR	31.2	>31.2	>62.5
AMP-C18-6	KRIWWR	15.6–31.2	>31.2	>62.5
AMP-C18-7	KIKRWWR	>31.2	>31.2	>62.5
AMP-C18-8	KIRWWR	>31.2	>31.2	>62.5
AMP-C18-9	RWR	>31.2	>31.2	>62.5
MP-C18-10	KIKRWR	>31.2	>31.2	>62.5
AMP-C18-11	KKIRWR	15.6–31.2	>31.2	>62.5
AMP-C18-12	KWK	31.2	>31.2	>62.5
AMP-C18-13	KKWK	31.2	31.2	31.2
AMP-C18-14	RIKWK	>31.2	>31.2	>62.5
AMP-C18-15	RWRW	>31.2	>31.2	>62.5
AMP-C18-16	KWKW	>31.2	>31.2	>62.5
AMP-C18-17	KKWW	>31.2	>31.2	>62.5

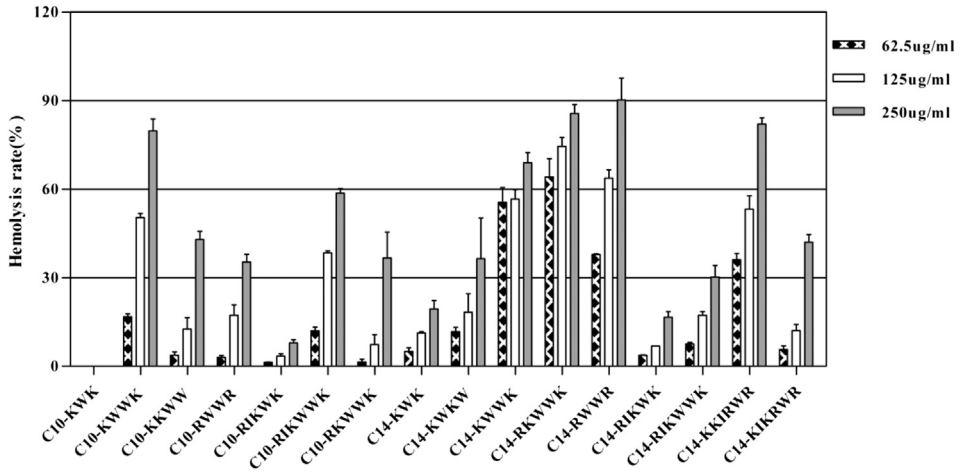

Fig. 1. Peptide hemolytic effect against fresh human erythrocytes. Each value is expressed as the mean of four independent replicates.

Table 4
Antimicrobial activity of RKWWK derivatives.

Name	MIC (μg/mL)	
	S. aureus	*E. coli*
AMP-C6-3	31.25	>62.5
AMP-C8-3	15.6	62.5
AMP-C10-3	3.9	3.9–7.8
AMP-C12-3	7.8	7.8
AMP-C14-3	7.8	15.6–31.2
AMP-C16-3	31.25	31.25
AMP-C18-3	31.25	125
RKWWK[a]	>62.5	>62.5
Rh-RKWWK[b]	15.6	>31.2

[a] RKWWK peptide only without lipid tail.
[b] N-terminal labeled with Rhodamine instead of lipid tail.

typical example is AMP-C14-12 (C14-KWK) and AMP-C14-1 (C14-KWWK), of which, the hemolytic effect at 125 μg/mL was 11% and 56%, respectively. However, 17% lytic effect of AMP-C14-16 (C14-KWKW) at the same concentration suggested that the hemolytic toxicity was also subject to the position of W residue. In addition, the toxicity data of AMP-C10-12 (C10-KWK) (<10% at 250 μg/mL) and AMP-C14-12 (C14-KWK) (19% at 250 μg/mL), as well as of AMP-C10-14 (C10-RIKWK) (<10% at 250 μg/mL) and AMP-C14-14 (C14-RIKWK) (20% at 250 μg/mL) indicated that the higher lytic effect was also associated with the longer lipid tail. Comparison of the toxicity of AMP-C10-3 (C10-RKWWK) (<10% at 125 μg/mL), AMP-C10-1 (C10-KWWK) and AMP-C10-4 (C10-RIKWWK) indicated that increase in charge would favor its discrimination ability between more neutral mammalian cell membranes and more negatively charged bacterial cell membranes. Overall, these data provided the evidences that hemolytic toxicity of peptides on human erythrocyte was tunable by varying their physicochemical parameters especially residual position, charge and hydrophobicity.

3.4. Structure and activity relationship study of lipopeptides

In this work, it is clear that the R, W and K containing lipopeptides yield potent antimicrobial activities. We then speculated how the structure and activity relationships of these nascent short peptides would be. Given the fact that the hydrophobicity is one of the major factors affecting the potency of antimicrobial activity, we specifically focused our attention on the hydrophobicity of C14 substituted congeners. The data were acquired from RP-HPLC analyses as shown in Table 1. All C14 substituted peptides were found to have a closely related hydrophobicity with the retention time from 10 to 13 min. The AMP-C14-5 was found to have a higher effective hydrophobicity, i.e., displayed longer retention time than the corresponding AMP-C14-9 with less one tryptophan residue, while the antimicrobial potency of AMP-C14-5 was two-fold lower than AMP-C14-9. The same relationship was also observed for peptide AMP-C14-1 and AMP-C14-2, in which the potency of AMP-C14-2 against *S. aureus* dropped four-fold owing to the increased hydrophobicity introduced by an additional isoleucine residue. However, the opposite effect was also observed for peptide AMP-C14-1 and AMP-C14-12, in which the more hydrophobic peptide of AMP-C14-1 achieved a higher potency.

To gain more insights into the relationships between antimicrobial activity and hydrophobicity, we then carefully selected a potent peptide of RKWWK and varied its length of lipid chain. The data in Table 4 shows that the most potent peptide was AMP-C10-3, while others with longer or shorter lipid chain were less potent. These data are consistent with our previous observations that a

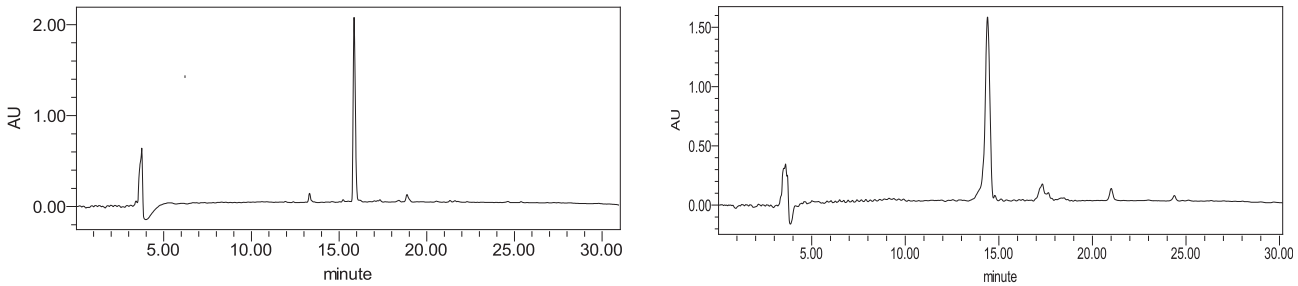

Fig. 2. HPLC profile of AMP-C10-3 (C10-RKWWK) and AMP-C10-14 (C10-RIKWK). Flow rate: 1 mL/min. Mobile phase: Solvent A: Water (0.1% trifluoroacetic acid), B: Acetonitrile (0.1% trifluoroacetic acid). Gradient:10%–15% B (2 min), 15%–35% B (6 min), 35%–50% B (10 min), 50%–70% B (7 min), and 70%–90% B (5 min).

potent antimicrobial peptide is a well balanced system and there exists an optimum hydrophobicity range in which the higher hydrophobicity is not always directly associated with the higher potency [9]. Decrease or increase hydrophobicity beyond this range will affect its antimicrobial potencies.

3.5. The mechanism of action of peptides is the disruption of integrity of bacterial membrane

Our next goal was to examine how the peptides exerted their function on bacteria. Generally, the mode of action for most

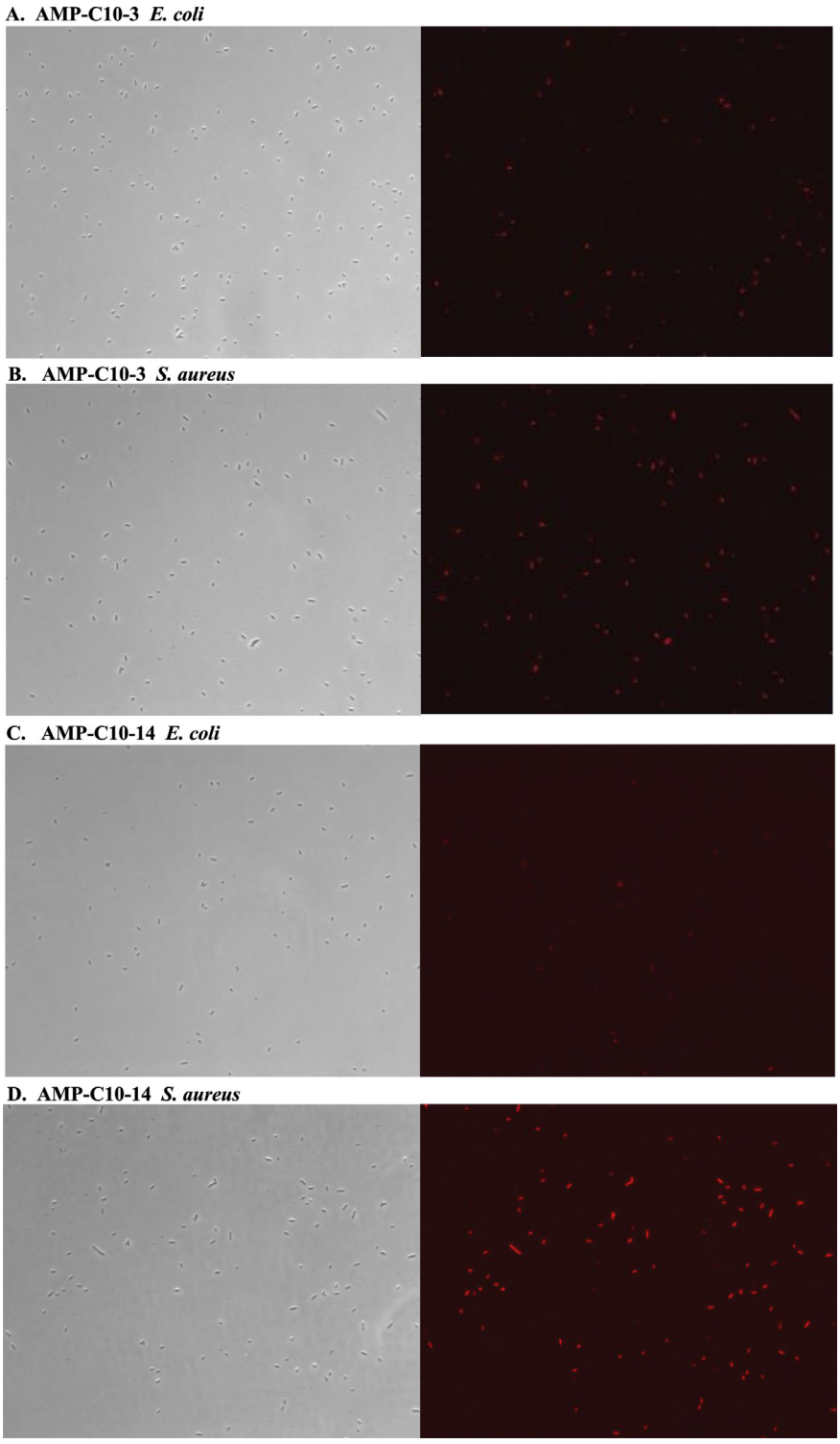

Fig. 3. PI influx assay of peptide AMP-C10-3 and AMP-C10-14 against *E. coli* and *S. aureus*. Two equally charged peptides of AMP-C10-3 (C10-RKWWK) and AMP-C10-14 (C10-RIKWK) were added into *E. coli* and *S. aureus* culture containing propidium iodide (PI), respectively. For AMP-C10-3 (**A** and **B**), the red fluorescence was visible toward both *E. coli* (**A**) and *S. aureus* (**B**) cells, while for AMP-C10-14 (**C** and **D**), the red fluorescence was much stronger from the interaction with *S. aureus* (**D**) than with *E. coli* (**C**) under the same condition. Bright-field (left column) and fluorescence (right column) images of the same field were acquired after each bacterium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

antimicrobial peptides is believed to bind and destroy the integrity of bacterial membrane [17,18], which will allow leakage of cytoplasmic molecules and subsequently lead to cell death. To characterize the interaction between our peptides and bacterial membrane, we employed a membrane impermeant fluorescent probe dye of propidium iodide (PI) to visualize the interaction of PI with cytoplasmic macromolecule of bacterial DNA induced by AMPs. PI is generally excluded from viable cells, while binds to double stranded DNA by intercalating between base pairs. Therefore, it provides a rapid and reliable method for the staining of non-viable cells injured by AMPs.

We selected two equally charged peptides of AMP-C10-14 (C10-RIKWK) and AMP-C10-3 (C10-RKWWK) to compare their interactions with a bacterial membrane. The results showed that both peptides were able to cause peptide-induced membrane damage and induce the influx of PI (Fig. 3). Interestingly, although these two peptides possess the same bactericidal potencies, similar hydrophobicity measured by HPLC analyses (Fig. 2), and equal charge, they exhibited different cell selectivity toward *E. coli* under the same condition (Fig. 3). For peptide AMP-C10-3, after incubation with *E. coli* and *S. aureus* respectively for 20 min (50 μg/mL), the red fluorescence was visible from both gram positive and gram negative bacterial cells. In contrast, AMP-C10-14 exhibited a much stronger fluoresce from the interaction with *S. aureus* than with *E. coli* under the same condition (Fig. 3). Obviously, these data indicated that both AMP-C10-14 and AMP-C10-3 were able to disrupt the integrity of the cell membrane and to influx the PI dye, however, the selectivity toward different cell membranes was different, which may involve the different mechanisms in the killing of bacteria.

3.6. Peptide AMP-C10-3 involves the interaction with intracellular molecules

In addition to the disruption of integrity of the bacterial membrane, the observation that AMP-C10-14 and AMP-C10-3 showed different disruption potential toward different cell membranes indicated that these two peptides may involve different mechanisms in cell killing. We then investigated that whether membrane disintegration was the sole mechanism by which these peptides to kill bacteria or the peptides had various intracellular targets. Several potent peptides were tested for their DNA-peptide binding ability by monitoring the electrophoretic mobility of plasmid DNA on an agarose gel. As shown in Fig. 4, peptide AMP-C10-3, AMP-C10-4 and AMP-C10-16 apparently exhibited DNA-binding ability by retarding the migration of DNA on agarose gel, whereas no interaction for AMP-C10-14 was observed at both 25 and 50 μg/mL.

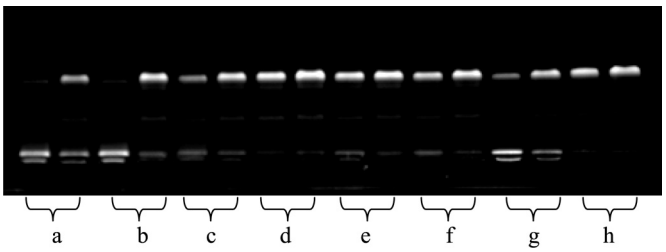

Fig. 4. DNA binding assay. From left to right, the numbers below the lanes represent the corresponding sample at 50 and 25 μg/mL, respectively: a: AMP-C10-3 (C10-RKWWK), b: AMP-C10-4 (C10-RIKWWK), c: AMP-C10-14 (C10-RIKWK), d: AMP-C10-17 (C10-KKWW), e: AMP-C10-5 (C10-RWWR), f: AMP-C10-10 (C10-KIKRWR), g: AMP-C10-16 (C10-KWKW), h: Methanol.

3.7. Peptide AMP-C10-3 is more toxic towards prokaryotes than towards mammalian cells

To further assess the potential of these peptides to cause cellular toxicity and evaluate their selectivity towards prokaryotic and mammalian cells, we tested the cytotoxicity of AMP-C10-3 (C10-RKWWK) and RKWWK alone without lipid chain against human TZM-b1 cells. The percent of survival is shown in Fig. 5. Apparently, AMP-C10-3 induced cell death at 125 μg/mL with CC_{50} at 115 μg/mL, whereas this toxicity did not occur for peptide RKWWK alone even at a concentration as high as 500 μg/mL (the highest concentration used). The similar trends were also observed for other peptides such as AMP-C10-4 (C10-RIKWWK) and AMP-C10-14 (C10-RIKWK), as well as against other different mammalian cell lines (data not shown) suggesting that these peptides would be safe if used in their MIC range, and the possible mechanism of cytotoxicity of these lipopeptides would result from a direct disruption of cell membrane, which would be improved by modulating the physicochemical properties of these peptides.

The cytotoxicity via MTT assay was assessed by incubation of cells with peptides over 48 h. To further address the discrimination ability of these peptides within a short period of time, we then treated the mammalian and bacterial cells with AMP-C10-3 and AMP-C10-14 respectively for only two hours. The viability of TZM-b1 cells was measured by flow cytometry analysis, while the survival of *S. aureus* was monitored by directly plating on MH agar plates. To our surprise, dramatic differences were observed that more than 99% of eukaryotic cells were untouched in two hours (Fig. 6A and B), in contrast, over 95% of *S. aureus* cells were eliminated by both AMP-C10-3 and AMP-C10-14 within only one hour (Fig. 6C) at the same peptide concentration. These data exhibited a differential ability of these peptides toward prokaryotes and mammalian cells, especially within a short period of time.

4. Discussion

Due to the wide spread use of conventional antibiotics in human, livestock and agriculture, antibiotic resistance has become a great concern in the world, prompting an urgent need to discover new classes of antibiotics with a different mechanism of action. Small antimicrobial peptides have been proposed as promising candidates and an alternative therapeutic option for pathogenic microorganisms owing to the small size, chemical ease of synthesis, low immune response, and unique mode of action. In this regard, R, K and W are represented as the core elements in generating novel AMPs with high microbicidal potencies. Nerveless, apart from their positive charge and hydrophobic bulk, the detailed roles of these amino acids in antimicrobial peptides are yet to be fully

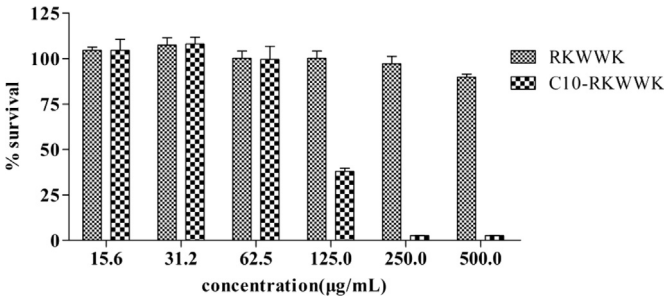

Fig. 5. Cytotoxicity of AMP-C10-3 (C10-RKWWK) and RKWWK. Cytotoxicity of AMP-C10-3 (C10-RKWWK) and RKWWK alone against human HeLa cells (TZM-b1) was evaluated by MTT assay. Each data is expressed as the mean of three independent replicates.

understood. An explanation to this is that the formation of cation–π electron interactions between the negatively charged electron cloud of indole residue and various cationic amino acid residues, such as R and W will contribute their additional effects to disrupt the integrity of bacterial membrane [15]. Based on this progress, in the present work, without taking into account the secondary structure, we demonstrate a series of short lipopeptides with a simple composition of R, K, W and I to search for potent antimicrobials. Our previous research has shown that a potent antimicrobial peptide is a well balanced system governed by its physicochemical properties especially by the hydrophobicity, charge and amphiphilicity [9]. Therefore, the potency of antimicrobial activity and selectivity toward different microorganisms are tunable by modulating these parameters, e.g. increasing the charge, changing the position of polar/apolar amino acids, changing the helicity, flexibility, or replacing hydrophobic/hydrophilic residues. To gain more insights into the role of the peptidyl hydrophobicity in antimicrobial activity, in this work, we have presented two strategies: (a) for different peptide sequences, the hydrophobicities were compared by reverse phase HPLC analyses, while (b) for the same sequence, the role of hydrophobicity was evaluated by connecting the peptidyl head with various fatty acid tail (C10, C14 and C18 fatty

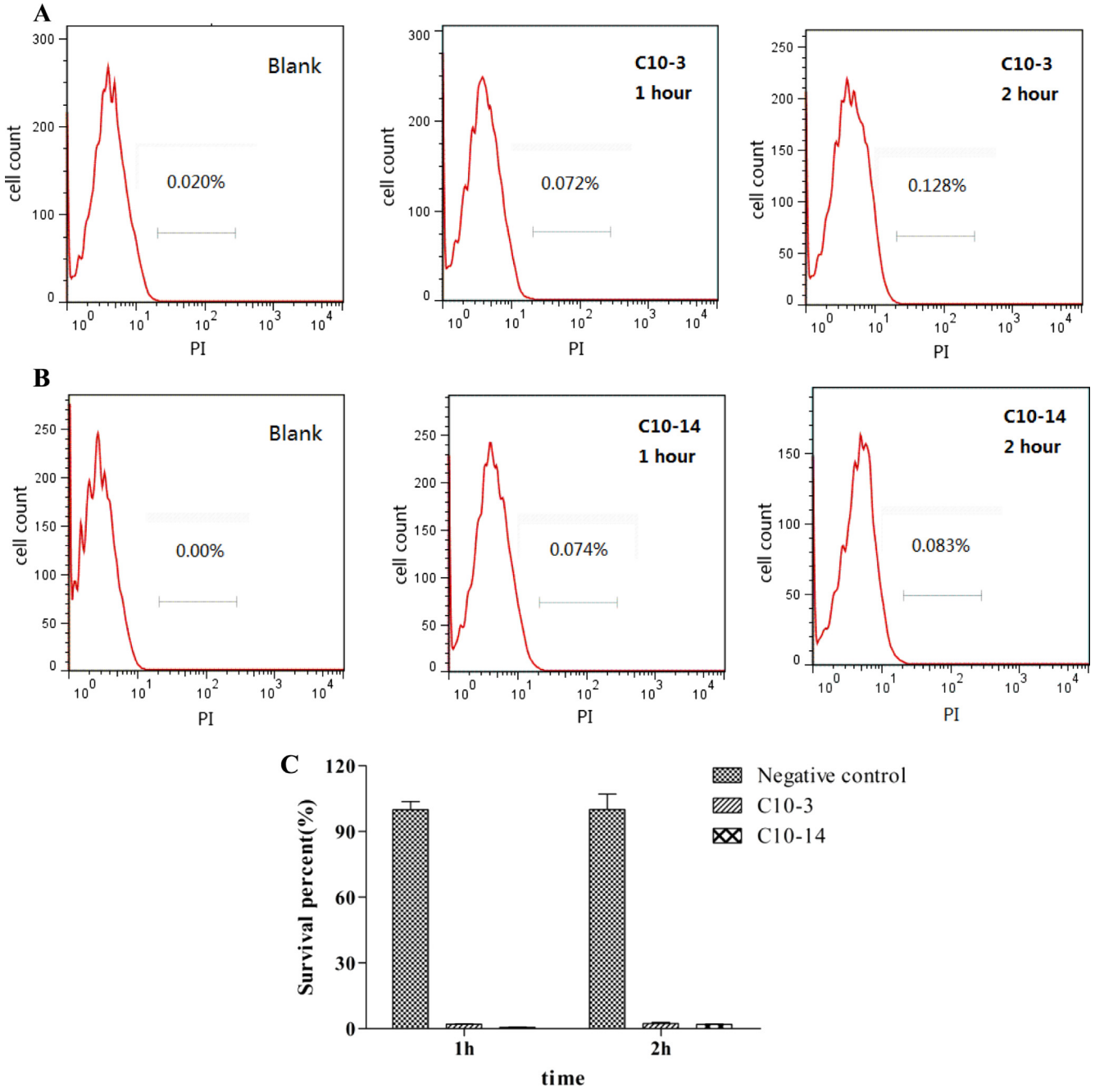

Fig. 6. Killing-kinetics of AMP-C10-3 and AMP-C10-14 against TZM-b1 cells (**A** and **B**), and *S. aureus* (**C**). The peptide concentrations were 40 μg/mL in 50% methanol/water, and the killing effects were monitored at one and two hours respectively. For TZM-b1 cells, both AMP-C10-3 (**A**) and AMP-C10-14 (**B**) killed less than 0.5% cells analyzed by flow cytometry analyses, while for *S. aureus* cells, over 95% cells were eliminated within one hour by both peptides. The negative control is the same volume of solvent alone (50% MeOH).

acids, respectively). On the basis of these experiments, a conclusion can be drawn that a potent antimicrobial peptide is a well balanced system and there exists an optimum hydrophobicity range in which high antimicrobial potencies could be achieved [19]. As a result, most peptides substituted with C18 fatty acid are less potent than their C14 and C10 congeners. The reason to this phenomenon is that a peptide with high hydrophobicity is prone to self-association, which prevents it from passing through the cell wall in microbial cells [20,21].

These small antimicrobial peptides possessing common biophysical characteristics, such as net positive charge, hydrophobicity, and amphiphilicity, are compatible with a mechanism of action that involves cellular properties like membrane perturbation and permeabilization. Recently, an increasing number of peptides are being described that act also on intracellular targets [14,22]. Here, we provide an evidence for peptide AMP-C10-14 (C10-RIKWK) and AMP-C10-3 (C10-RKWWK) that although these two peptides showed similar potencies with similar composition, the bacterial killing mechanisms may be different from each other. Apart from the common characteristics of membrane disruption ability detected with the PI dye, a DNA-staining fluorescent probe, AMP-C10-3 also involves action on intracellular targets like DNA-binding ability suggesting the multifunctional modes of action. However, it is not clear what the detailed mechanism might be upon the peptides entering into the bacterium, for example, they lyse the membrane or leave the membrane structure intact then to meet their intracellular targets. Furthermore, the interaction of peptides with DNA may involve the control of many cellular processes including DNA replication, recombination and repair, and transcription, we are also not clear the specific role of the peptide to interact with DNA. Besides their antimicrobial action, are any other functions of these AMPs to influence processes which support antimicrobial action involved, like cytokine release, chemotaxis, antigen presentation, and angiogenesis? Further studies including clinical observations are under way to analyze the individual contributions in more detail.

In conclusion, this study shows that R, K and W containing lipopeptides have a wide spectrum and potent antimicrobial activity against both gram positive and negative bacteria. Interestingly, several peptides such as AMP-C10-3 and AMP-C10-4 demonstrated multiple modes of action in their bacterial killing mechanism in addition to the membrane destabilization potential. Some of these synthetic lipopeptides are one of the shortest among all known antimicrobial peptides. It is easy to synthesize and cost effective. Therefore, these peptides have potential to develop as new therapeutic agents for a variety of infectious diseases.

Acknowledgments

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