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ORIGINAL ARTICLE

Poly-lysine peptidomimetics having potent antimicrobial activity without hemolytic activity

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Abstract Diversity of sequence and structure in naturally occurring antimicrobial peptides (AMPs) limits their intensive structure–activity relationship (SAR) study. In contrast, peptidomimetics have several advantages compared to naturally occurring peptide in terms of simple structure, convenient to analog synthesis, rapid elucidation of optimal physiochemical properties and low-cost synthesis. In search of short antimicrobial peptides using peptidomimetics, which provide facile access to identify the key factors involving in the destruction of pathogens through SAR study, a series of simple and short peptidomimetics consisting of multi-Lys residues and lipophilic moiety have been prepared and found to be active against several Gram-negative and Gram-positive bacteria containing methicillin-resistant *Staphylococcus aureus* (MRSA) without hemolytic activity. Based on the SAR studies, we found that hydrophobicity, +5 charges of multiple Lys residues, hydrocarbon tail lengths and cyclohexyl group were crucial for antimicrobial activity. Furthermore, membrane depolarization, dye leakage, inner membrane permeability and time-killing kinetics revealed that bacterial-killing mechanism of our peptidomimetics is different from the membrane-targeting AMPs (e. g. melittin and SMAP-29) and implied our peptidomimetics might kill bacteria via the intracellular-targeting mechanism as done by buforin-2.

Keywords Short peptidomimetics · Antimicrobial activity · Hemolytic activity · Structure–activity relationship (SAR) study · Poly-Lys peptidomimetics

Abbreviations

AMPs	Antimicrobial peptides
TFA	Trifluoroacetic acid
$DiSC_35$	3,3′-Dipropylthiadicarbocyanine iodide
ONPG	*O*-nitrophenyl-β-galactoside

M. Ahn and B. Jacob contributed equally to this work.

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MALDI-TOF MS	Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry
RP-HPLC	Reverse-phase high-performance liquid chromatography
CFU	Colony-forming unit
MIC	Minimal inhibitory concentration
LUVs	Large unilamellar vesicles
MRSA	Methicillin-resistant *Staphylococcus aureus*

Introduction

In the past few decades, antibiotics have gained significant importance as drug substances in pharmaceutical area in view of their biological importance in the critical fight against infectious disease caused by microorganisms. (O'Connell et al. 2013; Bush et al. 2011; Lewis 2012). However, excessive and irresponsible use of antibiotics results in the steady rise of drug-resistant bacteria called as "superbugs" which are an imminent and serious threat to global health care (Zasloff. 2002). For instance, methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA) are a form of *Staphylococcus aureus* that has developed resistance to the most clinically usable antibacterial drugs (Alonso and Garcia-del Portillo 2004). Antimicrobial peptides (AMPs) are considered as a universal feature of defense systems that are present in plants, amphibians and mammals. AMPs are produced by innate immune system, which delivers a firm action against invading foreign bodies such as bacteria, viruses, or fungi (Ganz 2003; Bals and Wilson 2003; Kim et al. 2011). In the modern health systems, AMPs are finding significant attentions as a new generation of antibiotics because of their broad spectrum of antimicrobial activity against pathogens at physiological concentration, their low propensity for the development of resistance, and their ability to kill multidrug-resistant bacteria (Hancock and Scott 2000). In spite of these advantageous features, their large size limits the advancement as therapeutic agents because of the high production cost, vulnerability to protease degradation, potential immunogenicity and cytotoxicity to red blood cells (Makovitzki et al. 2006; Chongsiriwatana et al. 2008; Sharma et al. 2009; Nguyen et al. 2010; Findlay et al. 2013; Hernandez-Gordillo et al. 2014). These conventional issues drive us to develop short peptidomimetics having unnatural residue without losing the cationic amphiphilic structure: cations interact with negatively charged microbial surfaces and hydrophobic groups permit incorporation into microbial membrane (Murugan et al. 2013a; Ahn et al. 2013). To develop short peptidomimetics, it is essential to study structure–activity relationship which can reveal the possible features of antimicrobial agents responsible for the destruction of bacteria. Unfortunately, diversity in AMP's sequence, structure and chain length limits the intensive SAR study (Tossi et al. 2000). In contrast, peptidomimetics have several advantages compared to naturally occurring peptide in terms of simple structure, convenient to analog synthesis, rapid elucidation of optimal physiochemical properties and low-cost synthesis. This work forms a part of our research program recently embarked on the synthesis of short and simple component of peptides as potent antimicrobial agents (Murugan et al. 2013b). In this work, we synthesized short antimicrobial peptidomimetics comprising of two components namely hydrophobic moieties and positively charged Lys residues. Our designed peptidomimetics consist of di- to penta-residues. Interestingly, despite their short peptide chain length and simple component, some of peptidomimetics showed the similar activity compared to many classical AMPs in terms of broad spectrum of antimicrobial activities and prokaryotic selectivity. For systematic screening of the antimicrobial and hemolytic activities of our peptidomimetics, we synthesized a series of analogs, which are bearing the diversity in structural features such as aromatic group including indole ring, positive charge, length of hydrocarbon tail and cyclohexyl group. To identify the most promising AMPs as a drug candidate, our designed peptidomimetics were examined and compared in terms of broad spectrum of antimicrobial activity, hemolytic activity and anti-MRSA activity. In addition, the bacterial killing mechanism of our designed peptidomimetics was elucidated using membrane depolarization, dye leakage, inner membrane permeability, and time-killing kinetics.

Materials and methods

Materials

Rink amide 4-methylbenzhydrylamine (MBHA) resin and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem–Novabiochem (La Jolla, CA). Other reagents used for peptide synthesis included trifluoroacetic acid (TFA: Sigma), piperidine (Merck), 1-O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU: Aldrich), N-hydroxybenzotriazole hydrate (HOBt: Aldrich), N,N-diisopropylethylamine (DIEA: Aldrich) and dimethylformamide (DMF, peptide synthesis grade: Biolab). RAW264.7 cells were purchased from American Type Culture Collection (Bethesda, MD). Phosphatidylethanolamine (PE, from egg yolk), phosphatidylglycerol (PG, from egg yolk), 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl-2H-tetrazolium bromide (MTT) and calcein were purchased from Sigma Chemical Co. (St. Louis, MO). $DiSC_3$-5 was obtained from Molecular Probes (Eugene, OR). Gramicidin D and ONPG were supplied by Sigma Chemical Co. (St. Louis, MO, USA). DMEM and FBS were supplied by HyClone (SeouLin, Bioscience, Korea). *Escherichia coli* ML-35, a lactose permease-deficient strain with cytoplasmic β-galactosidase activity, utilized for inner membrane permeability assays, was obtained from Prof. Jae Il Kim, Gwangju Institute of Science and Technology (GIST), Republic of Korea. All other reagents were of analytical grade. The buffers were prepared in double-glass-distilled water.

Synthesis and characterization of peptidomimetics

All peptidomimetics were prepared by Fmoc SPPS methods using Rink amide resin (100 mg) with an initial loading of 0.61 mmol/g, unless otherwise noted. Fmoc-Lys (Boc)-OH and Fmoc-Lys (Fmoc)-OH were purchased from Novabiochem. Other carboxylic acid derivatives were purchased from Sigma-Aldrich. Resins were swollen in DMF for 45 min prior to synthesis. For sequence extension, the Fmoc-protected amino acid (5 eq.) was activated by treatment with HBTU (5.0 eq.), HOBt (5.0 eq.) and DIEA (10 eq.) in DMF (2 mL, 0.15 mM) for 2 min. This solution was added to the free amine on resin, and the coupling reaction was allowed to proceed for 1 h with Vortex stirring. After washing with DMF, Fmoc deprotection was achieved with 20 % piperidine in DMF (1×10 min, 2×3 min). The resin was washed with DMF (3×3 min), and the process was repeated for the next amino acid. Carboxylic acid derivatives were coupled with N-term Lys using HBTU (5.0 eq.), HOBt (5.0 eq.) and DIEA (10 eq.) in DMF (2 mL, 0.15 mM) for 1 h with Vortex stirring and finally the resin was washed with DMF, methanol, dichloromethane and ether, and then dried under vacuum. Linear peptides were cleaved from the resin with 5 % triisopropylsilane (TIS) and 5 % H_2O in trifluoroacetic acid (TFA, ~2 mL of TFA per 100 mg of resin) for 2 h. The cleavage cocktail was mixed with cold ether to precipitate the peptide and then centrifuged. Preparative reverse-phase HPLC analysis (RP-HPLC) was carried out on the Vydac C_{18} column (15 μm, 20 mm × 250 mm) using 0–90 % water/acetonitrile gradient in the presence of 0.05 % TFA. The final purity of the peptides (>95 %) was assessed by RP-HPLC on an analytical Vydac C_{18} column (4.6 mm × 250 mm, 300 Å, 5 μm particle size). The molecular masses of purified peptides were determined using matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (KBSI, Ochang).

Antimicrobial activity (MIC)

The antimicrobial activity of the peptides against three Gram-positive bacterial strains and three Gram-negative bacterial strains was examined by the broth microdilution method in sterile 96-well plates. Aliquots (100 μL) of a bacterial suspension at 2×10^6 CFU/mL in 1 % peptone were added to 100 μL of the peptide solution (serial two-fold dilutions in 1 % peptone). After incubation for 18–20 h at 37 °C, bacterial growth inhibition was determined by measuring the absorbance at 600 nm with a Microplate Autoreader EL 800 (Bio-Tek Instruments, VT). The minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration that causes 100 % inhibition of microbial growth. Three types of Gram-positive bacteria [*Staphylococcus epidermidis* (KCTC 1917) *Bacillus subtilis* (KCTC 3068) and *Staphylococcus aureus* (KCTC 1621)] and three types of Gram-negative bacteria [*Escherichia coli* (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637) and *Salmonella typhimurium* (KCTC 1926)] were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Methicillin-resistant *Staphylococcus aureus* (MRSA) (CCARM 3089, CCARM 3090 and CCARM 3095) were obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University (Seoul, Korea). MIC values were determined from three independent experiments performed in triplicate.

Measurement of hemolytic activity

Hemolytic activity of peptides was tested against human red blood cells (hRBCs). Fresh human red blood cells (hRBCs) were centrifuged, washed three times with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4), dispensed into 96-well plates as 100 μL of 4 % (v/v) hRBC in PBS, and 100 μL of peptide solution was added to each well. Plates were incubated for 1 h at 37 °C, and then centrifuged at 1000 × g for 5 min. Samples (100 μL) of supernatant were transferred to 96-well plates and hemoglobin release was monitored by measuring absorbance at 414 nm. Zero hemolysis was determined in PBS (A_{PBS}) and 100 % hemolysis was determined in 0.1 % (v/v) Triton X-100 (A_{triton}). The hemolysis percentage was calculated as: $\%\ \text{hemolysis} = 100 \times [(A_{sample} - A_{PBS})/(A_{triton} - A_{PBS})]$

Cytotoxicity against mammalian cells

Cytotoxicity of the peptides against Mouse fibroblastic NIH-3T3 was determined by the MTT assay. NIH-3T3

cells were cultured in DMEM with 10 % FBS and were maintained under 5 % CO_2 at 37 °C. The cells were seeded on 96-well microplates at a density of 1×10^5 cells/well in 150 μL of DMEM containing 10 % FBS. The plates were then incubated for 24 h at 37 °C in a 5 % CO_2 atmosphere. 20 μL of peptide solution was added, and the plates were incubated for a further 2 days. Wells containing cells without peptides served as controls. Subsequently, 20 μL of an MTT solution (5 mg/mL) was added to each well, and the plates were incubated for a further 4 h at 37 °C. The precipitated MTT formazan was dissolved in 40 μL of 20 % (w/v) SDS containing 0.02 M HCl for 2 h. The absorbance at 570 nm was measured using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA). Percent cell survival was expressed as a percent ratio of A_{570} of cells treated with peptide over cells only.

Dye leakage assay

Calcein-entrapped LUVs composed of EYPE/EYPG (7:3, w/w) were prepared by vortexing the dried lipid in dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was subjected to 10 frozen–thaw cycles in liquid nitrogen and extruded 21 times through polycarbonate filters (two stacked 100-nm pore size filters) with a LiposoFast extruder (Avestin, Inc. Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. The concentration of calcein-entrapped LUVs was determined in triplicate by phosphorus analysis. Calcein leakage from LUVs was monitored at room temperature by measuring fluorescence intensity at an excitation wavelength of 490 nm and emission wavelength of 520 nm on a model RF-5301PC spectrophotometer. For determination of 100 % dye release, 20 μL 10 % Triton-X100 in Tris-buffer was added to dissolve the vesicles. The percentage of dye leakage caused by the peptides was calculated as follows: % Dye leakage $= 100 \times [(F - F_0)/(F_t - F_0)]$ where F is the fluorescence intensity achieved at 5 min after peptide addition, and F_0 and F_t represent fluorescence intensities without the peptides and with Triton X-100, respectively.

Membrane depolarization assay

The cytoplasmic membrane depolarization activity of the peptides was measured using the membrane potential-sensitive dye, $diSC_3$-5 as previously described. In brief, *Staphylococcus aureus* (KCTC 1621) grown at 37 °C with agitation to the mid-log phase ($OD_{600} = 0.4$) was harvested by centrifugation. Cells were washed twice with washing buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended into an OD_{600} of 0.05 in similar buffer. The cell suspension was incubated with 20 nM $diSC_3$-5 until stable reduction of fluorescence was achieved, implying incorporation of the dye into the bacterial membrane. Then KCl was added to a final concentration of 0.1 M to equilibrate K^+ levels. Membrane depolarization was monitored by recording changes in the intensity of fluorescence emission of the membrane potential-sensitive dye, $diSC_3$-5 (excitation $\lambda = 622$ nm, emission $\lambda = 670$ nm) after peptide addition. The membrane potential was fully dissipated by adding gramicidin D (final concentration of 0.2 nM). The membrane potential dissipating activity of the peptides is calculated as follows: % Membrane depolarization $= 100 \times [(F_p - F_0)/(F_g - F_0)]$. where F_0 denotes the stable fluorescence value after the addition of the $diSC_3$-5 dye, F_p denotes the fluorescence value 5 min after peptide addition, and F_g denotes the fluorescence signal after gramicidin D addition.

ONPG hydrolysis assay

Inner membrane permeability was determined by measurement in *E. coli* ML-35 of β-galactosidase activity using the normally impermeable, chromogenic substrate ONPG as substrate. *E. coli* ML-35 were washed in 10 mM sodium phosphate (pH 7.4) containing 100 mM NaCl and resuspended in the same buffer at a final concentration of $OD_{600} = 0.5$ containing 1.5 mM ONPG. The hydrolysis of ONPG to *o*-nitrophenol over time was monitored spectrophotometrically at 405 nm following the addition of peptide samples.

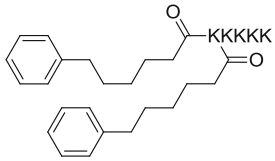
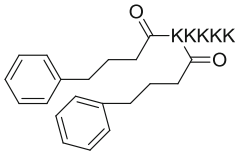
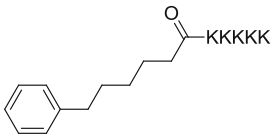
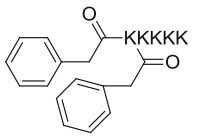
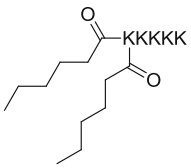
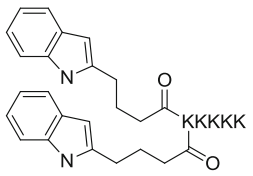
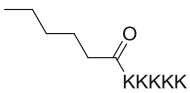
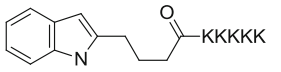
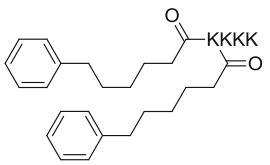
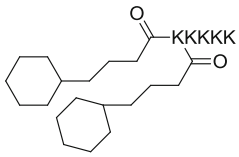
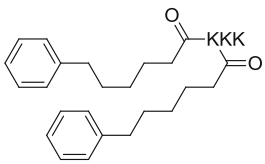
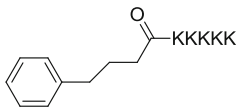
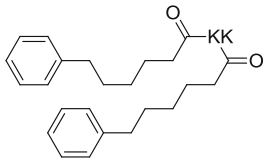
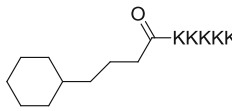
Time-killing kinetics

The time-killing kinetics of the peptides was assessed using *E. coli* (KCTC 1682). The initial density of the cultures was approximately 1×10^6 CFU/mL. After 1, 2, 5, 10, 20 or 40 min of exposure to the peptides at 37 °C, 50 μL aliquots of serial tenfold dilutions (up to 10^{-3}) of the cultures were plated onto Luria-Bertani (LB) agar plates to obtain viability counts. Colonies were counted after incubation for 24 h at 37 °C.

Results

We synthesized a series of short multi-Lys peptidomimetics. The N-term Lys residue is attached with various lipophilic moieties such as aromatic rings, cyclohexyl ring and linear aliphatic side chains at different lengths, which bring the diversity in structural features (Table 1; Fig. 1). Antimicrobial activities of all the peptides were assayed against three Gram-negative bacteria (*E. coli, P.*

Table 1 Synthetic peptidomimetics tested for their antimicrobial activity a: observed mass by MALDI-TOF MS b: calculated mass

No	Structure	M.W	No	Structure	M.W
K1		1008.4 [a] (1007.3)[b]	K8		952.5 (950.2)
K2		854.6 (853.2)	K9		895.6 (894.2)
K3		856.4 (855.2)	K10		1028.1 (1027.3)
K4		758.3 (757.2)	K11		844.2 (843.5)
K5		881.2 (879.7)	K12		963.5 (962.4)
K6		752.3 (751.0)	K13		805.1 (804.1)
K7		623.2 (622.4)	K14		811.2 (810.3)

aeruginosa and *S. typhimurium*) and three Gram-positive bacteria (*B. subtilis*, *S. epidermidis* and *S. aureus*). The cytotoxicity of the peptides was investigated by measuring their hemolytic activity toward human red blood cells (hRBCs) (Table 2).

Structure–Antimicrobial activity relationship study

The MICs obtained for the short peptidomimetics showed the different values according to the hydrophobicity, charge and length of hydrocarbon tail. It is gratifying to note that the several short peptidomimetics displayed activity with the MIC value of around 2–4 μg/mL against the bacterial strains (Table 2).

Hydrophobicity

Considering our interest to identify the effect of the hydrophobicity, we introduced di-phenyl (K1) and di-alkyl groups (K3) into the amine group of Lys. Further, removal

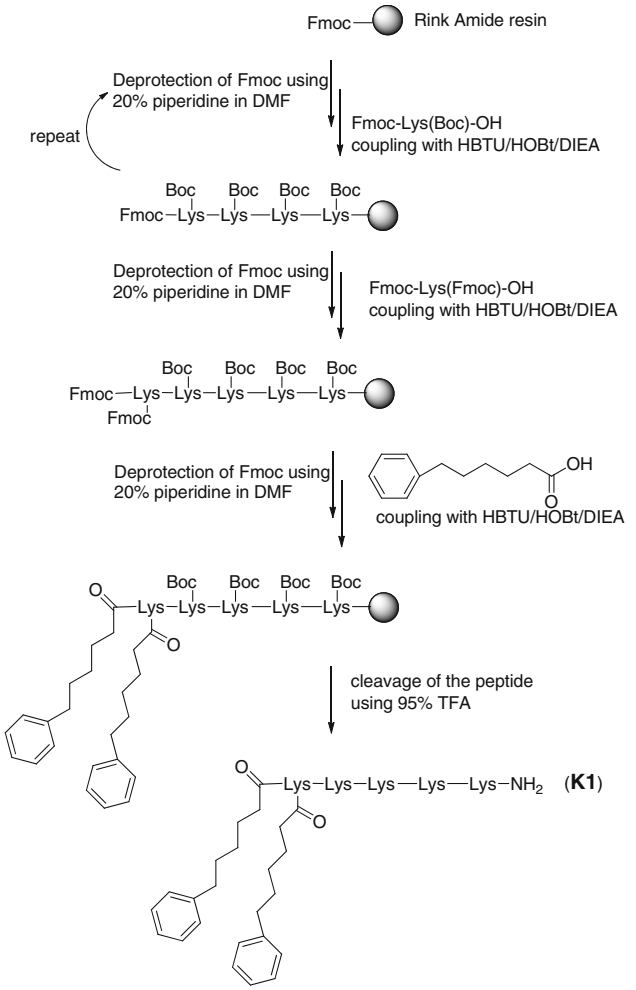


Fig. 1 Synthetic protocol of short peptidomimetic K1 using solid phase peptide synthesis (SPPS)

of phenyl alkyl chain from K1 resulted in K2 and truncation of one alkyl chain from K3 resulted in K4. Assay results revealed that K1 showed much better activity than K2 against all the bacterial strains except *S. typhimurium* and remains same in the case of *E. coli*. Further, K3 and K4 were analyzed and the results revealed that the K3 showed better activity than K4 against all the bacterial strains except *P. aeruginosa* and *S. aureus*. These results indicate that hydrophobicity has role in determining antimicrobial activity.

Charge

Presence of positively charged residues is believed to be an important factor for antimicrobial activity. As to understand the significances of charge in the antimicrobial activity, we designed peptidomimetics K5, K6 and K7 by the truncation of Lys residue at the C-terminus and compared with K1 as a standard for the antimicrobial activity. Encouragingly, K5 exhibited similar activity against all Gram-positive bacteria tested. In Gram-negative bacteria, *P. aeruginosa* showed same activity, while *E. coli* and *S. typhimurium* exhibited diminution of activity by two- and fourfold, respectively. This result undoubtedly implies that deletion of single Lys residue from K1 did not make significant difference in Gram-positive bacteria, while has a little effect on Gram-negative bacteria. Further, to support this hypothesis, it was important to design K6 by short of one Lys residue from K5. Unlike K5, the result of K6 was not in agreement with K5 as compared to K1. Peptidomimetic K6 displayed approximately a twofold decrease in the activity against Gram-negative and two- to fourfold diminution of activity of activity against Gram-positive bacteria except *S. aureus* which displayed similar activity, compared with the K5. However, there was no significant change in hemolytic activity of K6 even though there was a gain in the lipophilicity by deletion of a Lys residue. Further, to generalize the effect of charge in peptidomimetics, there was an urge of removing three Lys residues from K1 that resulted in K7. The results of K7 did not show any promising activity against both Gram-negative and Gram-positive bacteria in contrast displayed a significant hemolytic activity at 256 μg/mL. These results revealed that minimum number of Lys residues required for antimicrobial activity is three.

Length of hydrocarbon tail

It is believed that the variation in length of the long hydrocarbon linker has influence on the extent of antimicrobial activity. This hypothesis was verified by changing the length of the hydrocarbon linker between phenyl group and *N*-term Lys that resulted in the formation of C-5 (K1), C-3 (K8) and C-1 (K9). When there is change in carbon length by two numbers from K1 to K8, we found that the MIC value depends on the bacteria. In the case of Gram-negative bacteria, *E. coli* and *S. typhimurium* exhibited the increase in activity by twofold while *P. aeruginosa* showed the decrease in activity by twofold, when compared with K1. In Gram-positive bacteria, *S. epidermidis* and *S. aureus* exhibited the increase in activity by twofold while *B. subtilis* retained the activity compared with K1. Surprisingly, in the case of K9, four carbons lack in length from K1, displayed dramatic decrease in antimicrobial activity against both Gram-positive and Gram-negative bacterial stains. Thus above result showed that the carbon chain length has participation in determining antimicrobial activity, hence it is considered to be one of the key factors in designing the AMPs.

Table 2 Antimicrobial and hemolytic activities of synthetic antimicrobial peptidomimetics

Peptides	MIC (μg/ml)[a]						% Hemolysis (256 μg/ml)
	Gram-negative bacteria			Gram-positive bacteria			
	E. coli (KCTC 1682)	*P. aeruginosa* (KCTC 1637)	*S. typhimurium* (KCTC 1926)	*B. subtilis* (KCTC 3068)	*S. epidermidis* (KCTC 1917)	*S. aureus* (KCTC 1621)	
K1	8	8	8	2	2	4	0
K2	8	32	2	8	8	64	0
K3	32	>64	64	16	64	>64	0
K4	>64	>64	>64	>64	>64	>64	0
K5	16	8	32	2	2	4	0
K6	32	16	64	4	8	4	0
K7	>64	64	>64	32	64	64	44.8
K8	4	16	4	2	4	8	0
K9	16	>64	32	32	>64	>64	0
K10	16	16	16	4	16	32	0
K11	>64	>64	64	>64	>64	>64	0
K12	4	8	4	2	2	2	0
K13	32	>64	16	>64	>64	>64	0
K14	4	64	2	4	8	32	0

[a] MIC (minimal inhibitory concentration), the lowest peptide concentration for no microbial growth, was determined from three independent experiments performed in triplicate

Aromatic groups vs Cyclohexyl group

Since K8 showed the potent activity against both bacterial strains, we investigated the effect of phenyl group on antimicrobial activity by replacing phenyl group with cyclohexyl and indole group. Considering our interest in the designing of AMPs having heteroaromatic units such as indole group by replacing the phenyl motif in K8 motivated us to synthesize K10 and K11. Interestingly, K10 displayed a two- to fourfold decrease in activity against both bacterial stains, on the other hand K11 lost their antimicrobial activity against the both the bacterial stains compared to K8. Next our attention was turned over to compare the influence of cyclohexyl group in the design of AMPs. Subsequently, we synthesized the K12-anchoring cyclohexyl group at the expense of phenyl group as in K8. The assay results were very promising and most effective compared to other entities listed (Table 2). This result proved that the phenyl group was dispensable in the designing of most potential antimicrobial agents. With our continuing interest, K13 was synthesized from K8 by the removal of one phenyl alkyl chain. But, contrary to our expectations, K13 did not showed activity against any of the tested Gram-positive bacteria. Compared to K8, K13 was less active against Gram-negative bacteria, four fold and eight fold decrease against *S. typhimurium* and *E. coli*, respectively. K13 was inactive to *P. aeruginosa*. In addition, to identify the functional behavior of cyclohexyl group, we synthesized K14 from K13 by the replacement of phenyl group. The assay result showed that K14 recovered antimicrobial activity. Considering all these results, it was presumed that the cyclohexyl group was crucial for designing the potent AMPs.

Antimicrobial activity against MRSA

In addition to the broad-spectrum antimicrobial activity described above, next we evaluated the anti-MRSA activity against MRSA 1, 2 and 3. The assay results revealed that the MRSA activity depends on both lengths of hydrocarbon tail and linked functional groups (Table 3). For instance, the carbon chain length remains to be C-5 for K1 showed the anti-MRSA activity employed for all MRSA strains. However, the removal of two carbons from K1 remains as K8, which failed to display any encouraging activities at the tested concentration. To find the effect of functional group participation on anti-MRSA activity, we examined both K10 and K12 which are having the similar sequence with K8, except indole and cyclohexyl groups that are replacing phenyl group in K8. The assay results are very interesting since K12 disclosed the most potent anti-MRSA activity and K10 was not effective compared to K12. As seen above, our simple and short peptidomimetics made possible to analyze the systematic comparison and finally leads to these findings.

Table 3 Antimicrobial activity of synthetic antimicrobial peptidomimetics against methicillin-resistant *Staphylococcus aureus* (MRSA) strains

Peptides	Minimal Inhibitory Concentration (MIC) (μg/ml)		
	MRSA 1(CCARM 3089)	MRSA 2 (CCARM 3090)	MRSA 3 (CCARM 3095)
K1	4	4	4
K8	>32	>32	32
K10	>32	>32	>32
K12	4	4	4

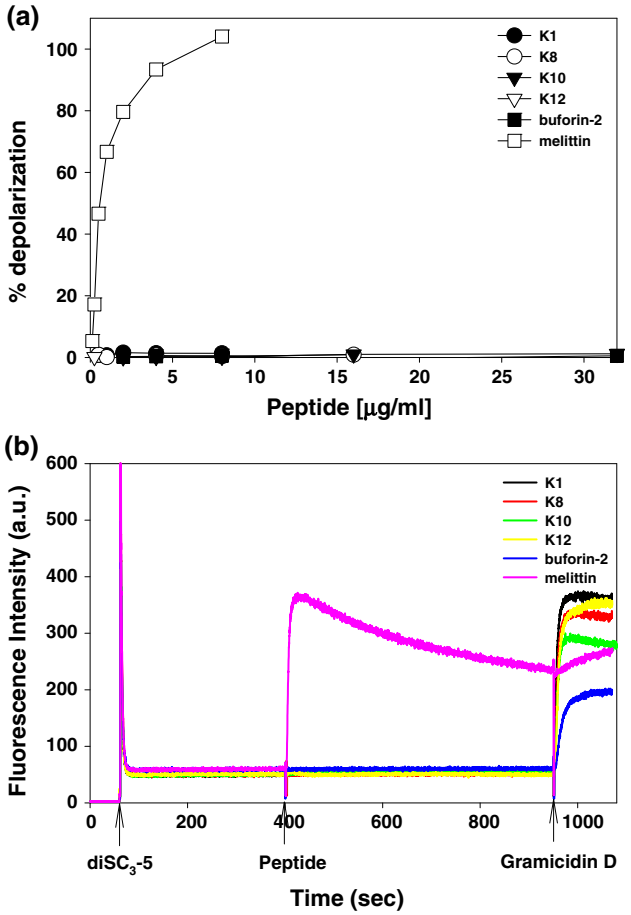


Fig. 2 **a** Concentration-dependent membrane depolarization of *Staphylococcus aureus* by the peptides. **b** Time-dependent membrane depolarization of *Staphylococcus aureus* by K1 (8 μg/mL), K8 (16 μg/mL), K10 (64 μg/mL), K12 (4 μg/mL), buforin-2 (32 μg/mL) and melittin (8 μg/mL)

Antimicrobial mechanism study

To investigate the plausible mechanistic action of our designed antimicrobial peptidomimetics, we were in urge to perform the following biochemical experiments.

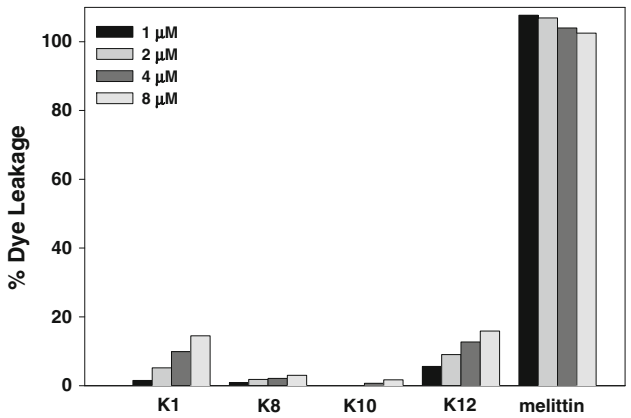


Fig. 3 Concentration-dependent peptide-induced dye leakage from calcein-entrapped negatively charged EYPE/EYPG (7:3, w/w) LUVs

Membrane depolarization

We used dye $diSC_3$-5 to monitor the cytoplasmic membrane depolarization of *S. aureus* cells. $DiSC_3$-5 was dispersed between the cells and medium, depending on the cytoplasmic membrane potential, and self-quenched when concentrated inside bacterial cells. We could easily measure the released fluorescence into medium if the membrane was depolarized. Melittin and buforin-2 were used as control because melittin is well known to induce the membrane depolarization, while buforin-2 causes no or less membrane depolarization (Turner et al. 1998). The experimental results revealed that our four compounds (K1, K8, K10 and K12) displayed the same pattern as buforin-2 which suggested that these compounds did not induce the membrane depolarization and mode of action was different from melittin (Fig. 2).

Dye leakage

Next, we tested the ability of peptidomimetics to induce the leakage of the fluorescent dye calcein entrapped within the large unilamellar vesicles (LUVs) composed of negatively charged phosphatidylethanolamine (PE)/phosphatidylglycerol(PG) (7:3, w/w). Melittin was used as control and induced 100 % leakage even at 1 μM concentration. In contrast, our four peptidomimetics caused <15 % leakage even at 8 μM (Fig. 3).

Inner membrane permeability

Inner membrane permeability is one of the critical steps for peptide to translocate into inner membrane. Inner membrane permeabilization is indicated by influx of nonchromogenic substrate ONPG that was subsequently

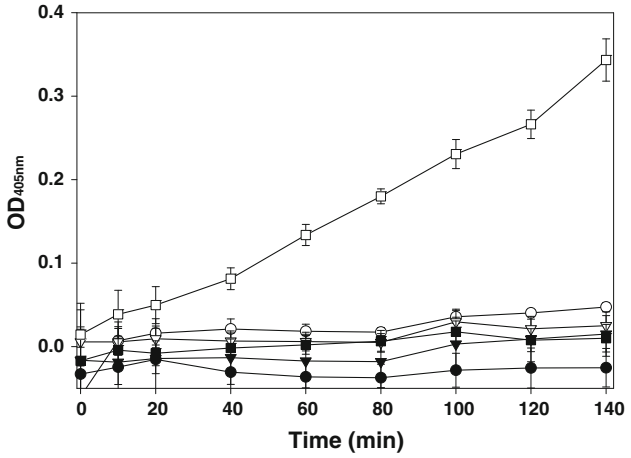


Fig. 4 Peptide-mediated inner membrane permeabilization of *Escherichia coli* ML-35. Permeabilization was determined by following spectrophotometrically at 405 nm, the unmasking of cytoplasmic β-galactosidase activity as assessed by hydrolysis of the normally impermeable, chromogenic substrate ONPG. *E. coli* ($\sim 10^6$ colony-forming units/ml) was resuspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate. Symbols: K1 (*black circle*), K8 (*white circle*), K10 (*black down-pointing triangle*), K12 (*white down-pointing triangle*), buforin-2 (*black square*) and SMAP-29 (*white square*)

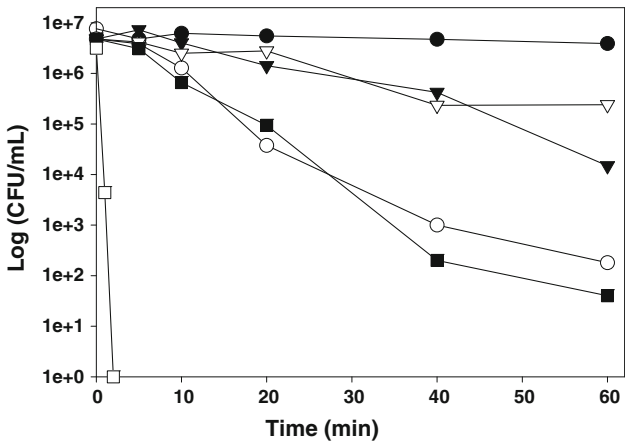


Fig. 5 Time-killing kinetics of *Escherichia coli* treated with the peptides at 2.0 × MIC. Symbols: without peptide (*black circle*), K1 (*white circle*), K8 (*black down-pointing triangle*), K10 (*white down-pointing triangle*), K12 (*black square*) and SMAP-29 (*white square*)

cleaved to yellow product ONP by β-galactosidase in the cytoplasm. Buforin-2 and SMAP-29 were used as reference peptides since Buforin-2 is well known for not inducing the inner membrane permeation while SMAP-29 has the ability to induce (Jacob et al. 2014). It is evidenced from Fig. 4, our peptidomimetics did not induce the inner membrane permeation even at two times MIC concentration.

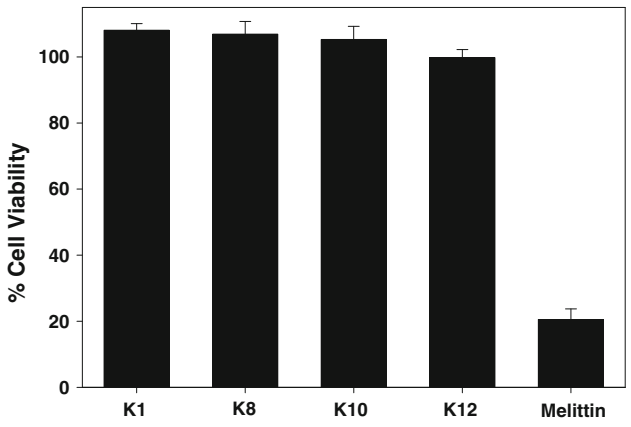


Fig. 6 Cytotoxicity of the peptides against mouse fibroblastic NIH-3T3 cells. The concentration of K1, K8, K10 and K12 is 50μg/mL. The concentration of melittin is 20μg/mL

Time-killing kinetics

To study how fast our compounds kill *E. coli*, their time-killing kinetics were tested (Fig. 5). While SMAP-29 used as reference peptide to kill the *E. coli* within 2 min, our peptides killed the target relatively very slow.

Cytotoxicity against mouse fibroblastic NIH-3T3 cells

The cytotoxicity of the peptides in mouse fibroblastic NIH-3T3 cells was evaluated by a standard MTT assay, which demonstrates active energization of cells and is conventionally used as a measure of cell viability. Melittin, a well-known cytolytic peptide, induced a remarkable cytotoxicity at 20 μg/mL, whereas K1, K8, K10 and K12 at 50 μg/mL were non-toxic to NIH-3T3 cells that resulted in nearly 100 % cell viability (Fig. 6).

Discussion

As there is increasing need of new antimicrobial substances for therapeutic agents, due to enormous development of microbial drug resistance, it is important for the modern world to find the alternate powerful antimicrobial substances. Consequently, one has to know mechanism of killing the bacteria to develop further antimicrobial agents. Unfortunately, mechanistic investigations in AMP's are entirely complex due to the extended diversity of AMP's in terms of their sequence and structure. In this study, our primary focus was to synthesize potent antimicrobial agents comprising of simple components while retaining the classical AMPs properties in terms of broad-spectrum antimicrobial activity and selectivity. It is obvious that simple component AMPs make us to understand the exact

mechanism to kill pathogens very easily. In our studies, we investigated short and simple AMPs action toward antimicrobial activity via systematic tuning of the factors such as hydrophobicity, charge, hydrocarbon tail lengths and functional groups. Interestingly, this strategy worked very well, and some of our designed AMPs exhibited potent broad-spectrum antimicrobial activity.

As a result of structure–activity relationship in this series of peptidomimetics, we discovered several important things; (1) Phenyl group (K1) is preferred over to alkyl group (K3), which might be due to the more hydrophobicity of phenyl ring than alkyl group (2) Hydrocarbon tail lengths between phenyl group and Lys residue are very crucial to display antimicrobial activity (K8 vs K9). This result indicated that the balance between hydrophobicity and charge is one of the key factors in designing AMPs. (3) Aromatic groups including indole group are not mandatory for designing the potent antimicrobial active agents (K8, K10 vs K12). It is an important finding because indole group of Trp is very sensitive to light. This has been a big hurdle to develop the short Trp-rich AMPs into new antimicrobial therapeutics (Ando et al. 2010). According to our result, cyclohexyl group is very good candidate to replace the indole group in terms of potent antimicrobial activity and selectivity. In addition, cyclohexyl group is more resistant to light.

Further, our simple and short peptidomimetics were subjected to the anti-MRSA assay to investigate the potential influence over the therapeutic application. Among them, K1 peptide showed the strong anti-MRSA activity while K8 failed to show the any encouraging activity which infers that carbon length played important role in displaying anti-MRSA activity. Interestingly, anti-MRSA activity can be recovered by replacing phenyl group of K8 with cyclohexyl group (K12). However, replacing phenyl group with indole group (K10) was not able to recover the anti-MRSA activity. The results from antimicrobial to anti-MRSA activity indicate that cyclohexyl group was found to be a most promising candidate to design AMPs.

In addition, to explain the bacteria-killing mechanism, we carried out several mechanistic studies including membrane depolarization, dye leakage from bacteria membrane-mimicking liposomes, inner membrane permeability and time-killing kinetics. Based on the mechanistic studies, we propose that unlike melittin, our peptidomimetics showed the weak ability to induce dye leakage and ONPG hydrolysis. Similar to buforin-2, our designed peptidomimetics did not cause any membrane depolarization even at MIC concentration against *S. aureus,* while melittin led to significant membrane depolarization against *S. aureus*. Furthermore, our peptidomimetics showed the very slow slop to kill the bacteria while SMAP-29 completely killed the bacteria within 2 min. Considering all these findings, we suggest that bacterial killing mechanism of our peptidomimetics is very different from the membrane-targeting AMPs (e. g. melittin and SMAP-29), and they may kill bacteria via the intracellular-targeting mechanism like buforin-2.

In conclusion, we synthesized the simple and short peptidomimetics consisting of poly-Lys residues and hydrophobic compounds, which provided potent antimicrobial activity as well as anti-MRSA activity. These simple peptidomimetics allowed us to study the SAR by the systematic comparison of common structural parameters such as hydrophobicity, positive charge, chain length and functional groups which were most important for the understanding of how AMPs kill the pathogens. These information lend the way to design the new therapeutic agents targeting microorganism.

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Conflict of interest The authors have declared that there is no conflict of interest.

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