Effect of disulphide bond position on salt resistance and LPS-neutralizing activity of α-helical homo-dimeric model antimicrobial peptides

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To investigate the effects of disulphide bond position on the salt resistance and lipopolysaccharide (LPS)-neutralizing activity of α-helical homo-dimeric antimicrobial peptides (AMPs), we synthesized an α-helical model peptide ($K_6L_4W_1$) and its homo-dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M, and di-$K_6L_4W_1$-C) with a disulphide bond at the N-terminus, the central position, and the C-terminus of the molecules, respectively. Unlike $K_6L_4W_1$ and di-$K_6L_4W_1$-M, the antimicrobial activity of di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C was unaffected by 150 mM NaCl. Both di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C caused much greater inhibitory effects on nitric oxide (NO) release in LPS-induced mouse macrophage RAW 264.7 cells, compared to di-$K_6L_4W_1$-M. Taken together, our results indicate that the presence of a disulphide bond at the N- or C-terminus of the molecule, rather than at the central position, is more effective when designing salt-resistant α-helical homo-dimeric AMPs with potent antimicrobial and LPS-neutralizing activities. [BMB reports 2011; 44(11): 747-752]

INTRODUCTION

Antimicrobial peptides (AMPs) can be categorized into 4 major classes on the basis of their sequence and structural characteristics: (i) amphipathic α-helical linear peptides, (ii) linear peptides presenting a cyclic moiety formed by a disulphide bond at the C terminus, (iii) β-hairpin peptides stabilized by 2 or more disulphide bridges, and (iv) certain amino acid-rich linear peptides such as Pro, Arg, or Trp. The first class includes several hetero- or homodimeric AMPs such as halocidin (1), distinctin (2), dicynthaurin (3), cathelicidin CAP11 (4), and PMAP-36 (5), consisting of 2 peptide chains linked by a disulphide bond. These molecules do not share any sequence homology. And

these dimeric AMPs have shown a broad spectrum of antimicrobial activity, with a remarkable range of effects on Gram-positive and Gram-negative bacteria, yeast, and fungi.

Considering the position of the disulphide bond, halocidin and dicynthaurin, which are isolated from the hemocytes of the tunicate, *Halocynthia aurantium*, have a disulphide bond located near the N-terminus and in the central portion of the molecules, respectively. Distinctin from the tree frog *Phyllomedusa distincta* and cathelicidin CAP 11 from guinea pig neutrophils have a disulphide bond near the C-terminal portion of the molecules. The disulphide bond of PMAP-36 from pig myeloid is positioned at the C-terminus.

The salt (NaCl is the predominant salt *in vivo*) sensitivity of cationic AMPs is a major obstacle in their development as novel therapeutic agents. Although AMPs exhibit significant antibacterial activity *in vitro*, many peptides seem to lose this activity under physiological salt conditions. Salt sensitivity has been observed in several AMPs, including β-defensins, cecropins, indolicidins, gramicidins, bactenecins, and magainins (6-8). However, not all peptides are salt sensitive, and some peptides show potent salt-insensitive antimicrobial activities (e.g. clavanin, tachyplesins, and polyphemusins) (8, 9). It is possible to develop synthetic α-helical peptides that substantially vary in activity and salt resistance by altering peptide hydrophobicity, amphipathicity, charge, and degree of α-helicity (10).

Recent studies have demonstrated that in addition to their antimicrobial activities, several AMPs including human LL-37, rabbit CAP18, sheep SMAP-29, bactenecin, indolicidin, and BMAP-27, have the potential to inhibit lipopolysaccharide (LPS)-induced cellular cytokine and/or nitric oxide (NO) release by directly binding to the LPS or by blocking the binding of LPS to the LPS-binding protein (LBP) (11,12). These properties render these peptides attractive drug candidates for the treatment of endotoxin shock and sepsis caused by infection with Gram-negative bacteria.

In the present study, to investigate the effects of the position of the disulphide bond located in homo-dimeric α-helical AMPs on salt resistance and LPS-neutralizing activity, we designed and synthesized an ideal amphipathic α-helical 11-meric model peptide ($K_6L_4W_1$) composed of 6 lysine molecules, 4 leucine

molecules, and 1 tryptophan molecule, and its 3 homo-dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M and di-$K_6L_4W_1$-C), with a disulphide bond at the N-terminus, the central position and the C-terminus of the molecules, respectively. The antimicrobial activity and bactericidal kinetics of these newly designed peptides against Gram-positive and Gram-negative bacterial strains were examined in the presence or absence of physiological levels of NaCl. The haemolytic activity of these peptides against human erythrocytes was also examined. Next, we investigated the mode of bactericidal action of the peptides by measuring their potential to cause the leakage of a fluorescent dye from lipid vesicles, the depolarization of the cytoplasmic membrane potential of *Staphylococcus aureus*. Furthermore, the LPS-neutralizing activity of these peptides was established by examining the inhibition of NO release in LPS-induced mouse macrophage RAW264.7 cells. Taken together, our results will help in designing salt-resistant α-helical homo-dimeric AMPs with potent LPS-neutralizing and antimicrobial activities.

RESULTS AND DISCUSSION

Synthesis of 3 dimeric peptides with a disulphide bond
Each corresponding monomeric peptide (CKLKKLWKKLLK-NH$_2$, KLKKLWCKKLLK-NH$_2$ and KLKKLWKKLLKC-NH$_2$) of the 3 dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M, and di-$K_6L_4W_1$-C)

Table 1. Amino acid sequences and calculated and observed molecular masses of the designed α-helical homo-dimeric model antimicrobial peptides

Peptides	Amino acid sequences	Molecular mass (Da)	
		Calculated	Observed
$K_6L_4W_1$	KLKKLWKKLLK-NH$_2$	1,424.9	1,424.8
di-$K_6L_4W_1$-N	(CKLKKLWKKLLK-NH$_2$)$_2$	3,054.0	3,053.3
di-$K_6L_4W_1$-M	(KLKKLWCKKLLK-NH$_2$)$_2$	3,054.0	3,052.9
di-$K_6L_4W_1$-C	(KLKKLWKKLLKC-NH$_2$)$_2$	3,054.0	3,053.3

was purified to >95% purity by reverse-phase high-performance liquid chromatography (RP-HPLC) on an analytical Vydac C_{18} column. The 3 homo-dimeric peptides were prepared by oxidation of each of the corresponding monomeric peptides. The oxidation of each monomeric peptide was performed in 10% DMSO solution under oxygen atmosphere for 48 h at room temperature (peptide concentration, 1 mg/ml). The disulphide bond formation of each monomeric peptide while forming the 3 homo-dimeric peptides was monitored by RP-HPLC on an analytical C_{18} column (data not shown). The final purity of the 3 homo-dimeric peptides, as analyzed by analytical RP-HPLC, was >95%. The correct molecular masses of the purified dimeric peptides were confirmed using matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan) (Table 1).

Antimicrobial and haemolytic activities
We examined the antimicrobial activities of the peptides against a representative set of bacterial strains, including 3 Gram-negative bacteria (*Escherichia coli* [KCTC 1682], *Pseudomonas aeruginosa* [KCTC 1637], and *Salmonella typhimurium* [KCTC 1926]) and 3 Gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917], and *Staphylococcus aureus* [KCTC 1621]). The MIC values are shown in Table 2. All the peptides showed similar effective MIC values against all the bacterial strains. The monomeric peptide $K_6L_4W_1$ and the 2 dimeric peptides (di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C) had similar MIC values in the range of 6.25-25 μg/ml against 6 different bacteria. In contrast, the antimicrobial activity of di-$K_6L_4W_1$-M was nearly 2-fold lower than that of the monomer $K_6L_4W_1$ (Table 2). Our results are in agreement with other studies that have shown that the dimerization in CAP11, PMAP-36, distinctin, and dicynthaurin does not affect the MIC values of the peptides (3, 13-15). However, the dimeric peptide halocidin showed a 4-20-fold increase in permeabilization and antimicrobial activity compared to a monomeric peptide (16). The disulphide-linked dimeric peptide of LLP1, derived from a lentivirus envelope protein, possesses much greater antimicrobial activity against *S. aureus* as compared to monomeric LLP1 (17). In addition, disulphide-

Table 2. Antimicrobial and hemolytic activities and cell selectivity of the designed model antimicrobial peptides

Peptide	MIC[a] (μg/ml)						GM[b] (μg/ml)	MHC[c] (μg/ml)	Therapeutic Index[d] (MHC/GM)
	E. coli	*P. aeruginosa*	*S. typhimurium*	*B. subtilis*	*S. epidermidis*	*S. aureus*			
$K_6L_4W_1$	12.5	25	6.25	12.5	12.5	6.25	12.5	400 <	64
di-$K_6L_4W_1$-N	12.5	12.5	12.5	6.25	12.5	12.5	11.5	140	12.2
di-$K_6L_4W_1$-M	25	25	12.5	12.5	25	25	20.8	400 <	38.5
di-$K_6L_4W_1$-C	12.5	12.5	6.25	6.25	12.5	12.5	10.4	47	4.5

[a]MIC were determined in three independent experiments performed in triplicate. [b]The geometric mean (GM) of the MIC values from all six bacterial strains in this table. [c]The minimal peptide concentration (MHC) that produces 10% hemolysis. When no detectable hemolysis was observed at 400 μg/ml, we used a value of 800 μg/ml to calculate the therapeutic index. [d]The ratio of the MHC (μg/ml) over the geometric mean (GM) of the MIC (μg/ml).
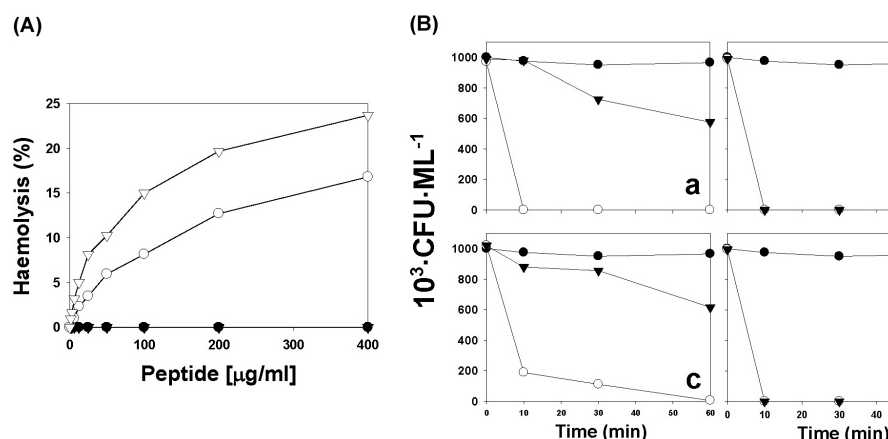
(A)

(B)

linked dimers of magainin 2 induced membrane permeabilization at lower concentrations than the monomeric form (18). These results suggest that the disulphide bond in various dimeric-AMPs seems only to partly contribute to their microbicidal effects. We next assessed the haemoytic effects of these peptides against mammalian cells by measuring their ability to cause lysis of human erythrocytes. The concentration-response curves for the haemolytic activity of the peptides are shown in Fig. 1A. For a quantitative measure of the haemolytic activity of the peptides, we introduced the minimal haemolytic concentration (MHC) defined as the lowest peptide concentration that produced 10% haemolysis (Table 2). The MHC values for di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C were 140 µg/ml and 47 µg/ml, respectively. However, these 2 peptides did not induce significant haemolysis at their MIC values against the bacterial strains. $K_6L_4W_1$ and di-$K_6L_4W_1$-M did not cause haemolysis at the highest peptide concentration tested (400 µg/ml).

Therapeutic index

The therapeutic potential of AMP drugs lies in the ability of the peptides to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. This property is defined by the concept of the therapeutic index (TI) as a measure of the relative safety of the drugs (19, 20). The TI of each peptide was calculated as the ratio of the MHC value to the geometric mean (GM) of MICs against 6 selected microorganisms (Table 2). When haemolysis was significantly absent at the highest concentration tested (400 µg/ml), 800 µg/ml was used for the calculation of TI, since the test was carried out by a 2-fold serial dilution. A high TI is thus an indication of two preferred characteristics of the peptide, namely, a high MHC (low haemolytic activity) and a low MIC (high antimicrobial activity) value. All the dimeric peptides had a lower TI than the monomeric peptide because of their increased haemolytic activity. Among the dimeric peptides, di-$K_6L_4W_1$-M had the highest TI. The order of TI for the peptides was $K_6L_4W_1$ > di-$K_6L_4W_1$-M > di-$K_6L_4W_1$-N > di-$K_6L_4W_1$-C.

Salt resistance

For effective use in clinical pharmacotherapy, AMPs need to remain active in the presence of physiological levels of salt (120–150 mM NaCl). To evaluate the effect of the position of the disulphide bond in the homo-dimeric α-helical AMPs in terms of their salt resistance, we examined the MICs of the peptides against 6 microorganisms and bactericidal kinetics at a concentration of 2 × MIC toward *E. coli* in the presence or absence of 150 mM NaCl. When judged in terms of its MIC value (Table 3), the monomeric peptide, $K_6L_4W_1$ displayed a 2-6-fold reduction in antimicrobial activity in the presence of 150 mM NaCl, as compared to its activity in the absence of NaCl. Two dimeric peptides, di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C, exhibited nearly unaltered antimicrobial activity against both Gram-positive and Gram-negative bacteria in the presence of 150 mM NaCl. In contrast, di-$K_6L_4W_1$-M did not show any antimicrobial activity even at the highest concentration tested (100 µg/ml). As shown in Fig. 1B, the bactericidal kinetics of di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C were not suppressed at 150 mM NaCl. In contrast, the bactericidal kinetics of $K_6L_4W_1$ and di-$K_6L_4W_1$-M were significantly inhibited at high salt concentrations. The salt resistance of di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C may be largely due to their multimeric oligomerization at high concentrations of NaCl. Lee *et al.* recently reported that the salt resistance of bactenecin homodimers is due to increased multimeric oligomerization at a high salt concentration (21). These results indicate that a disulphide bond at the N- or C-terminus of the molecule, rather than at the central position, is more effective with respect to antimicrobial activity when designing salt-resistant α-helical dimeric AMPs.

Mode of bactericidal action

To examine whether the antimicrobial activity of the peptides depends on their ability to permeate bacterial membranes, we measured their abilities to induce calcein leakage from negatively charged EYPE/EYPG (7 : 3 w/w) LUVs (bacterial cell membrane-mimicking environment). All of the peptides induced weak dye leakage of below 30% at 25 µg/ml (Fig. 2A).

Table 3. Antimicrobial activity of the designed model antimicrobial peptides in the presence of 150 mM NaCl

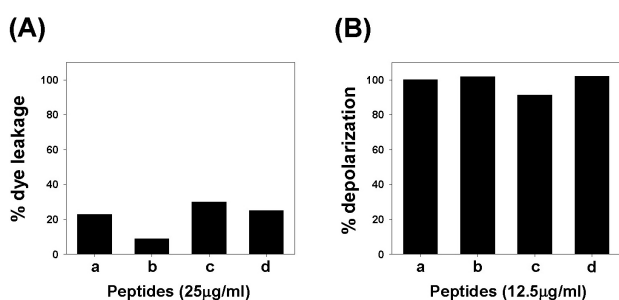| Peptide	MIC (µg/ml)					
	E. coli	*P. aeruginosa*	*S. typhimurium*	*B. subtilis*	*S. epidermidis*	*S. aureus*
$K_6L_4W_1$	100	100	100	25	50	100
di-$K_6L_4W_1$-N	12.5	12.5	12.5	6.25	12.5	12.5
di-$K_6L_4W_1$-M	100 <	100 <	100 <	100 <	100 <	100 <
di-$K_6L_4W_1$-C	12.5	12.5	6.25	6.25	12.5	12.5

Fig. 2. (A) Percent dye leakage from negatively charged EYPE/EYPG (7 : 3, w/w) LUVs measured at 2 min after the addition the peptides. The concentration of EYPE/EYPG (7 : 3, w/w) LUVs was 68 µM. (B) Percent membrane depolarization of *Staphylococcus aureus* (OD$_{600}$ = 0.05) by the peptides using the membrane potential sensitive dye, diSC$_3$-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Peptides: a ($K_6L_4W_1$); b (di-$K_6L_4W_1$-N); c (di-$K_6L_4W_1$-M); and d (di-$K_6L_4W_1$-C).
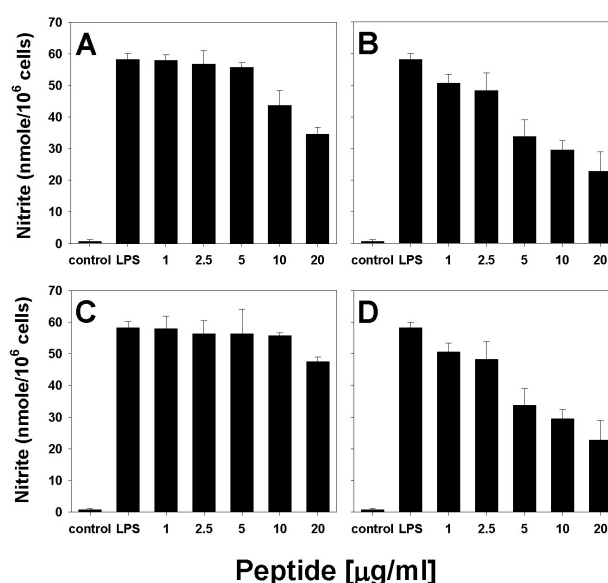

Peptide [µg/ml]

Fig. 3. Inhibitory activities of the peptides on LPS-stimulated nitric oxide (NO) production in RAW264.7 cells. The RAW264.7 cells (5 × 10^5 cells/ml) were treated with 20 ng/ml LPS in the absence or presence of various concentrations (1.0 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml and 20 µg/ml) of $K_6L_4W_1$ (A); di-$K_6L_4W_1$-N (B); di-$K_6L_4W_1$-M (C); and di-$K_6L_4W_1$-C (D). Error bars represent standard deviations of the mean determined from 3 independent experiments.

Next, to evaluate the effects of the peptides on *S. aureus* cytoplasmic membranes, the membrane potential-sensitive fluorescent dye diSC$_3$-5 was used. This dye is distributed between the cells and the medium, depending on the cytoplasmic membrane potential, and self-quenches when concentrated inside bacterial cells. If the membrane is depolarized, this dye will be released into the medium, causing a measurable increase in fluorescence. All of the peptides caused significant membrane depolarization of above 90% at 12.5 µg/ml (Fig. 2B). These findings support the channel/pore formation model as the mechanism of bactericidal action of our designed peptides.

LPS-neutralizing activity

Sepsis is the major cause of mortality in the intensive care unit, accounting for 200,000 deaths every year in the United States alone (22). Release of LPS from antibiotic-treated Gram-negative bacteria can indeed enhance sepsis (12). Therefore, an effective antimicrobial agent should not only exert antimicrobial activity but also have the ability to neutralize LPS. To investigate whether $K_6L_4W_1$ and its 3 dimeric peptides possess LPS-neutralizing activity, as well as potent antimicrobial activity, we assessed their ability to inhibit NO release in LPS-stimulated mouse macrophage RAW264.7 cells. $K_6L_4W_1$, di-$K_6L_4W_1$-N, and di-$K_6L_4W_1$-C significantly inhibited NO release in LPS-stimulated mouse macro-

phage RAW264.7 cells at concentrations of 20 µg/ml, 5 µg/ml, and 5 µg/ml, respectively (Fig. 3). In particular, both di-$K_6L_4W_1$-N and di-$K_6L_4W_1$-C showed much greater inhibition of NO release compared to di-$K_6L_4W_1$-M (Fig. 3). This result indicates that a disulphide bond at the N- or C-terminus of the molecule is more effective than at the central position when designing α-helical dimeric AMPs with potent LPS-neutralizing activity. Taken together, our results will be useful for designing novel dimeric salt-resistant AMPs with potent antimicrobial and LPS-neutralizing activities.

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), dicyclohexylcarbodiimide (DCC; Fluka), *N*-hydroxybenzotriazole hydrate (HOBT; Aldrich), and dimethylformamide (DMF, Biolab). Lipopolysaccharide (LPS, from *Escherichia coli* O111 : B4), egg yolk L-α-phosphatidylethanolamine (EYPE), egg yolk L-α-phosphatidyl-DL-glycerol (EYPG), and calcein were purchased from Sigma Chemical Co (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by HyClone (SeouLin, Bioscience, Korea). RAW264.7 cells were purchased from the American Type Culture Collection (Bethesda, MD). All other reagents were of analytical grade.

Peptide synthesis

The peptides listed in Table 1 were prepared using the standard Fmoc-based solid-phase synthesis technique on Rink amide MBHA resin. DCC and HOBt were used as coupling reagents, and a 10-fold excess of Fmoc-amino acids was added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/H_2O/thioanisole/phenol/ethanedithiol/triisopropylsilane (81.5 : 5 : 5 : 5 : 2.5 : 1, v/v) for 2 h at room temperature, the crude peptides consisting of the monomeric peptide, $K_6L_4W_1$, and corresponding linear peptides (CKLKKLWKKLLK-NH_2, KLKKLWCKKLLK-NH_2 and KLK KLWKKLLKC-NH_2) of three dimeric peptides (di-$K_6L_4W_1$-N, di-$K_6L_4W_1$-M and di-$K_6L_4W_1$-C), were repeatedly extracted with diethyl ether and purified by RP-HPLC on a preparative Vydac C_{18} column (15 μm, 20 × 250 mm) using an appropriate 0-90% water/acetonitrile gradient in the presence of 0.05% TFA. The molecular masses of the purified linear peptides were determined using MALDI-TOF MS (Shimadzu, Japan).

Antimicrobial activity (MIC)

All bacterial strains were supplied from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The antimicrobial activity of the peptides against three Gram-positive bacterial strains and three Gram-negative bacterial strains was examined by using the broth microdilution method in sterile 96-well plates. Aliquots (100 μl) of a bacterial suspension at 2 × 10^6 colony-forming units (CFU)/ml in 1% peptone with 0 or 150 mM NaCl were added to 100 μl of the peptide solution (serial 2-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 minimum peptide concentration inhibiting bacteria growth.

Bactericidal kinetics

The kinetics of the peptides' bactericidal activity was assessed using *E. coli* (KCTC 1682) and *S. aureus* (KCTC 1621) at MIC × 2 in the presence of 0 or 150 mM NaCl, as described in a previous study (23). The initial density of the cultures was approx-imately 1×10^5 CFU/ml. After 0, 10, 30, or 60 min of exposure to the peptides at 37°C, 50 μl aliquots of serial 10-fold 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.

Haemolytic activity

Fresh human red blood cells (hRBCs) were washed 3 times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.4) by centrifugation for 7 min at 1,000 × g and then resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 μl of hRBC suspension [4% (v/v) in final] in PBS to a final volume of 200 μl, and incubated for 1h at 37°C. The samples were centrifuged at 1,000 × g for 5 min, and hemoglobin release was monitored by measuring the supernatant absorbance at 405 nm with a Microplate ELISA Reader (Bio-Tek Instruments, VT, USA). The minimal haemolytic concentration (MHC) was defined as the minimal peptide concentration that produced 10% hemolysis. hRBCs in PBS (A_{blank}) or 0.1% Triton X-100 (A_{triton}) were used as negative and positive controls, respectively. The haemolysis percentage was calculated according to the equation:

$$\% \text{ haemolysis} = 100 \times [(A_{sample} - A_{blank}) / (A_{triton} - A_{blank})].$$

Dye leakage

Calcein leakage from vesicles was determined by measuring the decrease in self-quenching. The fluorescence intensities of calcein released from large unilamellar vesicles (LUVs) composed of EYPE/EYPG (7 : 3, w/w) were monitored at 520 nm (excitation at 490 nm) on a RF-5301PC spectrophotometer (Shimadzu, Japan) after 2 min of incubation with the peptide. The fluorescence intensity corresponding to 100% leakage was determined by the addition of Triton X-100 to the sample (finally 0.1%, v/v).

Membrane depolarization

The membrane depolarization activity of individual peptides was determined by the membrane potential-sensitive fluorescent dye, diSC$_3$-5. Briefly, *S. aureus* grown at 37°C with agitation to the mid-log phase ($OD_{600} = 0.4$) was harvested by centrifugation. The cells were washed twice with washing buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended to an OD_{600} of 0.05 in similar buffer containing 0.1 M KCl. Subsequently, the cells were incubated with 20 nM diSC$_3$-5 until stable reduction of fluorescence was achieved, implying incorporation of the dye into the bacterial membrane. An excitation wavelength of 622 nm and an emission wavelength of 660 nm were used to monitor depolarization. The membrane potential was fully dissipated by adding gramicidin D (finally 0.2 nM).

Measurement of nitric oxide (NO) release from LPS-induced RAW264.7 cells

RAW 264.7 cells were grown in DMEM supplemented with

10% fetal bovine serum and antibiotics in 5% CO_2 and 95% air at 37°C. Nitrite accumulation in the culture media was used as an indicator of nitric oxide (NO) release. The RAW264.7 macrophages were cultured overnight in 96-wells plate (5×10^5 cells/well). The medium was then removed followed by the addition of fresh DMEM supplemented with 5% of bovine serum to each well. The cells were stimulated with LPS (20 ng/ml) in the presence or absence of peptides. Cells that were stimulated with LPS alone and untreated cells served as controls. After incubating for 24 h, the amount of NO in the supernatant was estimated from the accumulation of the stable NO metabolite nitrite with Griess reagent according to the manufacturers' instructions (1% sulfanilic acid, 0.1% N-1-Naphthylethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 540 nm.

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