Biomaterials 34 (2013) 237–250

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Design of hybrid β-hairpin peptides with enhanced cell specificity and potent anti-inflammatory activity

YiFan Liu [a], Xi Xia [a], Liang Xu [b], YiZhen Wang [a,*]

[a] National Engineering Laboratory of Biological Feed Safety and Pollution Prevention and Control, Key laboratory of Molecular Animal Nutrition, Ministry of Education, Institute of Feed Science, Zhejiang University, 588 Yuhang Tang Road, Hangzhou 310058, China
[b] Cancer Science Institute of Singapore, National University of Singapore, Centre for Translational Medicine, 14 Medical Drive, #12-01, 117599 Singapore

ARTICLE INFO

Article history:
Received 30 August 2012
Accepted 16 September 2012
Available online 6 October 2012

Keywords:
Antimicrobial peptides
β-Hairpin
Antibacterial activity
Bactericidal mechanism
Hemolysis
Anti-inflammatory

ABSTRACT

Antimicrobial peptides (AMPs) have attracted considerable attention for their broad-spectrum antimicrobial activity and reduced tendency to cause bacterial resistance. Emerging concerns over the host cytotoxicity of AMPs, however, may ultimately compromise their development as pharmaceuticals. In order to optimize AMPs with potent cell specificity and anti-inflammatory activity, we designed β-hairpin hybrid peptides based upon progetrin-1, bovine lactoferricin and cecropin A. The synthetic hybrid peptides LB-PG and CA-PG demonstrated high selectivity over a wide range of microbes from Gram-positive and Gram-negative bacteria in porcine red blood cells. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) show that these peptides kill microbial cells by penetrating the cell membrane and damaging the membrane envelope. Gel retardation demonstrates that the peptides have a high affinity for DNA, indicating an additional possible intracellular bactericidal mechanism. Moreover, the hybrid peptides inhibit the expression of LPS-induced proinflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), macrophage inflammatory protein-1α (*MIP-1α*) and monocyte chemoattractant protein 1(MCP-1), following LPS stimulation in RAW264.7 cells. Our results indicate that these hybrid peptides have considerable potential for future development as antimicrobial and anti-inflammatory agents.

1. Introduction

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immunity of organisms which provide a foolproof mechanism for fighting invasion of various microbial pathogens, and are amongst the most promising candidates for future antibiotic drugs [1]. While these molecules have been reported to exist in random structures in their native forms, they often adopt different secondary conformations such as α-helices, β-sheets, linear or loop structures in membrane-like environments [2]. In spite of variations in lengths and primary structures, AMPs share several common features, such as: net cationic charge; amphiphilicity; primarily targeting cell membranes instead of specific receptors; and possessing antimicrobial activity through disruption of the microbial cell membrane [3–6]. The robust capability of AMPs to destroy microbial membranes suggests that they can potentially evade mechanisms involved in multidrug resistance. For example, Navon-Venezia et al. reported that repeated exposure of Gram-positive bacteria towards different AMPs at sub-minimum inhibitory concentrations did not alter the minimum inhibitory concentration (MIC) value significantly for up to 10–15 bacterial passages [7].

Even though natural AMPs have shown to be superior in overcoming multidrug resistance, issues such as being easily degradable, costliness and a lack of information about systemic toxicity are likely to be serious obstacles to further development of natural peptides as antibacterial drugs [8]. Nowadays, peptide-based biomaterials have gained much attention due to their ability to overcome such disadvantages and provide excellent templates for a wide range of bioengineering and biomedical applications, including regenerative medicines, biomimetic materials, therapeutic delivery and antimicrobial agents [9–12]. Various approaches have been employed to optimize the native sequence of AMPs, e.g., through modification and/or optimization of natural peptide/protein sequences, and are designed to improve antimicrobial effects whilst reducing undesirable cytotoxic effects in mammalian cells [13–16]. There remains however a lack of systematic scientific rationale in the design principles of AMPs.

* Corresponding author. Tel.: +86 571 88982815; fax: +86 571 88982650.
E-mail address: yzwang@zju.edu.cn (YiZhen Wang).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.09.032

Hybridization is one novel method to design peptides combining the advantages of different native peptides. Given their inherent amphiphilic character and ability to readily aggregate in bulk solution, these peptides show great potential at interfaces [17]. However, there is little research describing such design methods.

Most recently, we have compared the antibacterial activity, cytotoxicity and mechanisms of cathelicidin peptides and found that porcine protegrin-1(PG-1), a β-hairpin peptide, showed high antimicrobial potency but was also more toxic to eukaryotic cells [18]. Bovine lactoferricin (LfcinB), a 25-residue disulfide cross-linked peptide from the N-terminus of lactoferrin, possesses very low hemolytic activity towards porcine red blood even at concentration of 256 μg/mL [19]. Cecropin A (CA), one of the first reported AMPs, is found in the hemolymph of *Hyalophora cecropia* pupae and has the capacity to kill bacteria by making their cell membranes leaky, but has few effects on insect or mammalian cells [20]. In this study, we designed hybrid peptides by combining the 1–16 or 5–17 β-fold regions of protegrin-1 with the N-terminal cationic amphipathic region of cecropin A (1–8) or the active center of bovine lactoferricin (4–9). We speculate that with these modifications, the effects of amphiphilicity and charge on bactericidal capacity can be better conserved and the cytotoxicity of PG-1 can be reduced. The peptides were first characterized for their secondary conformation by circular dichroism (CD) in membrane-mimicking environments, such as sodium dodecyl sulfate (SDS) and trifluoroethyl alcohol (TFE). The antimicrobial properties of the peptides were studied using bacteriostatic MIC measurement against a broad selection of practically threatening microbes from Gram-positive bacteria: *S. aureus*, Gram-negative bacteria: *Escherichia coli*, *Salmonella*, and *Pseudomonas aerogenosa*. Scanning electron microscopy, transmission electron microscopy and DNA binding assays were employed to investigate potential membrane destruction mechanisms as well as possible intracellular targets of the peptides. The potential cytotoxic effect of peptides against mammalian cells was characterized by measuring their hemolytic effect on porcine red blood cells (pRBCs). To investigate whether these antimicrobial peptides show anti-inflammatory activities, peptide inhibition of TNF-α production and TNF-α, iNOS, *MIP-1α* and MCP-1 gene expression in LPS-stimulated mouse macrophage RAW264.7 cells were examined. Finally, the capability of the peptides to synergize with antibiotics was evaluated using the checkerboard titration method. The overall objective of the study is to facilitate the development of peptide-based strategies to treat microbial infection by regulating immune activity.

2. Materials and methods

2.1. Hybrid peptides design

We constructed the hybrid peptides by combining the 1–16 or 5–17 β-fold regions of protegrin-1 with the N-terminal cationic amphipathic region of cecropin A (1–8) or the active center of bovine lactoferricin (4–9). The amino acid sequences of the parental and hybrid peptides are listed in Table 1.

2.2. Sequence analysis of hybrid peptides

Primary sequence analysis of the peptides was performed by bioinformatics programs including ProtParam (ExPASy Proteomics Server: http://www.expasy.org/tools/protparam.html) and Pepstats (EMBOSS biology software: http://www.ebi.ac.uk/Tools/emboss/pepinfo/). The mean hydrophobicity, mean hydrophobic moment and relative hydrophobic moment were calculated online using CCS scale: http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html. The secondary structure type for each residue was predicted by PSIPRDE v3.0 (http://bioinf.cs.ucl.ac.uk/psipred/) and online by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The helical wheel projection and physicochemical profiles were calculated online using the Helical Wheel Projections: http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.

2.3. Peptides synthesis

Based on the calculated physicochemical parameters and the predicted secondary structures, the parental peptides protegrin-1, LfcinB and cecropin A, and the hybrid peptides LB(4–9)-PG(5–17) (LB-PG), CA(1–8)-PG(1–16) (CA-PG) were synthesized and purified by GL Biochem (Shanghai, China) and their molecular weights further confirmed via matrix-assisted laser desorption/ionization them-of-flight mass spectroscopy (MALDI-TOF MS, Model Autoflex, Bruker Daltonics Inc., U.S.A.), using α-cyano-4-hydroxycinnamic acid as the matrix. The purity of the peptides was determined at more than 95% with analytical reverse phase (RP)-HPLC. α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Sigma–Aldrich (Singapore) and used in saturated acetonitrile/water (1:1 volume ratio) after re-crystallization. Peptides were then dissolved in pure water at a concentration of 3.0 mg/mL and the peptide solutions were stored at −20 °C before subsequent antibacterial and structural assessments.

Aureomycin and neomycin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Stock solutions of each medicine (3.0 mg/mL) were prepared in endotoxin-free water, pH of 7.4, and stored in 20 μL aliquots at −20 °C until used.

2.4. CD analysis

To investigate conformational changes induced by membrane environments, peptides were dissolved at 0.1 mg/mL in deionized (DI) water, 25 mM SDS surfactant or 50% TFE. CD spectra were recorded at 25 °C on a JASCO J-815 spectropolarimeter fitted with a PTC-423S single position. The Peltier temperature controller and percent of each structure was calculated using a spectra manager. The secondary structure of the peptides was investigated at wavelengths ranging from 190 to 250 nm using a quartz cell with a path length of 0.1 mm using a 3.0-nm spectral bandwidth, 0.2 nm step resolution, 100 nm/min scanning rate and 2 s response time and averaged over 3 runs of each sample. The acquired CD signal spectra were then converted to mean residue ellipticity using the following equation:

$$\theta_M = (\theta_{obs}/10) \times (M_{RW}/c \cdot l)$$

where θ_M is residue ellipticity (deg. M^{-1} m^{-1}), θ_{obs} is the observed ellipticity corrected for the buffer at a given wavelength (mdeg), M_{RW} is residue molecular weight (M_W/number of amino acids), c is peptide concentration (mg/mL), and l is the path length (cm).

2.5. Measurement of antimicrobial activity (MIC)

The antimicrobial activities of peptides against seven Gram-negative bacterial strains, included isolated strains, and two Gram-positive bacterial strains were determined using an improved broth microdilution technique as previously described [21]. Briefly, serial diluted (4–256 μg/mL) aliquots of the peptides were

Table 1
Key physicochemical parameters of parental and hybrid peptides.

Peptides	Sequence	Theoretical M_W	Measured M_W[a]	Retention time (min)	H[b]	μHrel[c]	Net charge
PG-1	RGGRLCYCRRRFCVCVGR	2160.6	2156.7	8.87	−2.55	0.23	+6
LB	FKCRRWQWRMKKLGAPSITCVRRAF	3125.8	3123.8	9.85	−1.46	0.24	+8
CA	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	4004.8	4004.86	11.16	−1.37	0.22	+6
LB-PG	RRWQWRLCYCRRRFCVCVG	2547.1	2543.09	11.58	−1.46	0.27	+6
CA-PG	KWKLFKKIRGGRLCYCRRRFCVCV	3019.8	3015.80	12.61	−1.46	0.18	+9

[a] Measured by MALDI-TOF, apparent $M_W = [M_W + H]$.
[b] The mean hydrophobicity (H) is the total hydrophobicity (sum of all residue hydrophobicity indices) divided by the number of residues.
[c] The relative hydrophobic moment (μHrel) of a peptide is its hydrophobic moment relative to that of a perfectly amphipathic peptide. This gives a better idea of the amphipathicity using different scales. A value of 0.5 thus indicates that the peptide has about 50% of the maximum possible amphipathicity.

added to sterile 96-well polypropylene microtiter plates (cat. 3790, Costar, Cambridge, MA) in a volume of 10 μL, followed by the addition of 90 μL of diluted bacteria ($2–7 \times 10^5$ colony-forming units (CFUs)/mL; exponential phase). After incubation for 18–20 h at 37 °C, MICs were determined as the lowest peptide concentration at which no bacterial growth was observed. A negative control of pure broth without microbes was used for the MIC tests, each of which was reproduced 3 times using 6 replicates for each experiment.

Standard strains *E. coli* ATCC25922, *E. coli* K88, *E. coli* K12, *E. coli EPEC* O78:K88, *S. choleraesuis* CMCC50020, *S. typhimurium* CMCC50013, *Pseudomonas aeruginosa* CMCC27853, *S. aureus* ATCC25923, *S. epidermidis* ATCC12228 were purchased from China General Microbiological Culture Collection Center (Beijing, China). The strains above were grown in Mueller-Hinton (MH) broth (Difco, England), pH 6.8 at 37 °C. Probiotic bacteria, *Bifidobacterium suis* ATCC27533 (*B. suis*) and *Lactobacillus acidophilus* ATCC4356 (*L. acidophilus*) were also used, which were purchased from China General Microbiological Culture Collection Center (Beijing, China), and maintained at 37 °C under anaerobic conditions in Peptone Yeast Glucose (PYG) and de Man Rogosa Sharpe (MRS) media, respectively.

2.6. Antibacterial mechanism study

The mechanisms of action of the antimicrobial peptides were elucidated by direct observation of the bacterial membrane structure by scanning (SEM) and transmission (TEM) electron microscopy, as well as by indirect intracellular target observation using a DNA binding assay.

2.6.1. SEM characterization

For SEM sample preparation, bacterial cells were cultured in MH at 37 °C under constant shaking at 250 rpm. In this study, *E. coli* ATCC25922 and *S. aureus* ATCC25923 were cultured to an exponential phase, harvested by centrifugation, washed twice with 10 mM PBS and re-suspended. Approximately 2×10^7 cells were incubated at 37 °C for up to 60 min with different peptides at a concentration of 1 × MIC. Controls were run without peptides. After incubation, the cells were mixed and centrifuged at 5000 × g for 5 min. Bacterial pellets were then washed 3 times with PBS, and subjected to similar centrifugation after each wash. Following that, fixation of bacterial cells was performed with 2.5% (w/v) glutaraldehyde at 4 °C overnight, followed by washing with PBS twice. Cells were harvested and post-fixed with 1% osmium tetroxide for 1 h then dehydrated for 15 min in each of a graded ethanol series (50, 70, 80, 90, 95 and 100%). They were then transferred to absolute acetone for 20 min, a mixture ($v:v = 1:1$) of alcohol and iso-amyl acetate for 30 min, then pure iso-amyl acetate for 1 h. Finally, specimens were dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid CO_2. The dehydrated specimens were coated with gold-palladium and observed in a Philips Model XL30 ESEM.

2.6.2. TEM observations

Pretreatment with bacteria samples was conducted in the same way as for SEM treatment. After treatment with a series of ethanol solutions (50, 70, 80, 90, 95 and 100%) for 15 min each, bacterial cells were transferred to absolute acetone for 20 min. The specimens were then transferred to 1:3 and 1:1 mixtures of absolute acetone and resin for 1–3 h then to a final resin overnight. Finally, specimens were placed in capsules containing embedding medium and were heated at 70 °C for about 9 h. Specimens were stained by uranyl acetate and alkaline lead citrate for 15 min and observed by TEM (Model JEM-1230, JEOL, Japan).

2.6.3. DNA binding assay

Gel retardation experiments were performed by mixing 400 ng plasmid DNA (pGEM-βGAL; Promega, Madison, WI, USA) with increasing amounts of peptides in 20 μL binding buffer 5% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl and 50 μg/mL bovine serum albumin) as previously described by Park et al. and Ulvatne et al. [22,23]. The mixture was incubated for 1 h at room temperature. Subsequently, 4 μL of the native loading buffer (10% Ficoll 400, 10 mM Tris–HCl, pH 7.5, 50 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) was added to each vial and a 12 μL aliquot was applied to a 1% agarose gel, followed by electrophoresis in 0.5 × Tris borate–EDTA buffer.

2.7. Synergy with conventional antibiotics

The checkerboard titration method is widely used for evaluation of properties of different antibacterial combinations [24]. The fractional inhibitory concentration (FIC) index is used to determine the synergy between antimicrobial agents. An inoculum of logarithmic-phase bacteria ($2–7 \times 10^5$ CFU/mL) was cultured at 37 °C for 24 h in each well of a 96-well flat-bottomed culture plate containing 100 μL of MH broth with peptides or antibiotics, alone or in combination. Two-fold serial dilutions of aureomycin and neomycin were tested in the presence of a constant amount of peptide, equal to one-quarter of the peptide MIC. The FIC index was calculated as follows: FIC index = (MIC drug A in combination) / (MIC drug A alone) + (MIC drug B in combination) / (MIC drug B alone). An FIC index of less than 0.5 is considered to demonstrate synergy.

2.8. Measurement of hemolytic activity

The hemolytic activity of each peptide was evaluated by determining hemoglobin release from erythrocyte suspensions of healthy pigs (Large white , obtained from Zhejiang University, Hangzhou, China). The blood cells were washed three times in sterile phosphate buffer (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH7.4) and centrifuged at 370 × g for 10 min at room temperature. The 90 μL erythrocyte suspensions (final concentration around 1%) were then mixed with 10 μL of test peptides at different concentrations (4–256 μg/mL) and the mixtures were incubated at 37 °C under 5% CO_2 for 24 h. After incubation, plates were centrifuged at 370 × g for 5 min and supernatants were transferred into 96-well ELISA plates (cat. 3590, Costar, Cambridge, MA) and the optical density was measured at 576 nm using Multi-Mode Microplate Readers (SpectraMax M5, Molecular Devices, USA). Two control groups were provided for this assay: untreated pRBC suspension (as a negative control), and a pRBC suspension treated with 0.1% Triton-X (as a positive control). Each solution was tested in triplicate.

Hemolysis (%) = [(O.D.576 nm of the treated sample − O.D.576 nm of the negative control) / (O.D.576 nm of positive control − O.D.576 nm of negative control)] × 100%.

2.9. Resistance to proteolytic digestion

Sample preparation for the resistance to proteolytic digestion assay was carried out both with broth microdilution assay and reverse phase high-performance liquid chromatography (RP-HPLC) assay. (1) Broth microdilution assay. Ten microliters of artificial gastric juice (1 g porcine pepsin (Sigma P7012) or trypsin (Sigma P1750) added to 100 mL water) was used to dilute the peptides (stock solution) to yield a final peptide concentration of 2 × MIC. After incubation at 37 °C for 1 h, 10 μL of the mixture was added to 90 μL bacterial suspension ($2–7 \times 10^5$ CFU/mL) and incubated at 37 °C for overnight. Bacterial growth inhibition was determined by measuring absorbance at 600 nm with a Multi-Mode Microplate Readers (SpectraMax M5, Molecular Devices, USA). (2) RP-HPLC assay. RP-HPLC analysis was performed with Water-600 series HPLC system using a Zorbax SB C_{18} (250 × 4.6 mm I·D, 5 μm) column(flow = 1 mL/min, detection = 214 nm). Enzymatic stability was assessed with simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) individually. SGF was prepared as described in the Chinese Pharmacopoeia and consists of 10 mg/mL pepsin in 0.03 M NaCl at pH 1.5. SIF consists of 10 mg/mL of pancreatin in 0.05 M KH_2PO_4, pH 7.5. Nine microliters of SGF or SIF were added to 10 μL of peptides dissolved in distilled water at 3.0 mg/mL concentration. After incubation periods of 0, 5, 15, 30, 45 and 60 min at 37 °C, 1 μL of 0.1 M NaOH were added to SGF or 0.5% trichloroacetic acid (TFA) were added to the SIF. Sixty microliters of acetonitrile were added to the mixture, and centrifuged at 13,000 rpm for 10 min. The supernatant was submitted to a determination of the relative concentrations of the remaining peptides by RP-HPLC.

2.10. Cells and conditions

The murine macrophage cell line RAW264.7 was obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) and cultured in DMEM medium (Gibco) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen), 2 mM L-glutamine, and 10% fetal bovine serum (Gibco). Cells were grown at 37 °C and 5% CO_2 in humidified air and passaged every 2–3 days.

2.11. Cytotoxicity (WST-1 proliferation and LDH release assay)

The viability of peptide-treated RAW264.7 cells was determined using a WST-1 assay (Roche Molecular Biochemicals; Mannheim, Germany). Cells were pre-seeded on a 96-well plate at a density of 2×10^5 cells/mL in DMEM medium (supplemented with 10% heat inactivated FBS). Ten microliters of varying concentrations of peptide solution were added and cells were incubated for another 24 h at 37 °C in 5% CO_2. Wells containing untreated cells served as controls. Subsequently, 10 μL of WST-1 solution was added to each well and the plates were incubated for 2 h at 37 °C. The absorbance at 450 nm was measured using a Microplate autoreader (SpectraMax M5, Molecular Devices, USA).

The effect of peptides on plasma membrane integrity of RAW264.7 cells was examined using an LDH release assay (Roche Molecular Biochemicals; Mannheim, Germany). Cell supernatants were analyzed for LDH enzymatic activity, which under control conditions is exclusively intracellular. RAW264.7 cells (2×10^5 cells/well) were cultured for 24 h at 37 °C in 5% CO_2 in the absence or presence of peptides at concentrations ranging between 4 and 128 μg/mL. The maximal LDH release of cells was determined by addition of lysis solution (1% Triton X-100) at 37 °C for 15 min. Untreated cells served as controls for spontaneous LDH release. Absorbance was subsequently measured in a microtiter plate reader at 490 nm, using 900 nm as a reference wavelength. LDH release activity was expressed as the percentage of maximal LDH.

2.12. Effect of peptides on LPS-stimulated cytokine Induction

RAW264.7 cells were preincubated with or without 10 μg/mL peptides for 30 min before the addition of 100 ng/mL LPS (*E. coli*, O55:B5, Sigma–Aldrich,

Germany) and further incubation for 5 h at 37 °C. After stimulation, the cells were detached from the wells and washed once with ice-cold phosphate-buffered saline. Total RNA was extracted using TRI® reagent according to the manufacturer's instructions. The quality and quantity of the RNA samples were determined by NANODROP 2000 Spectrophotometer (Thermo, USA). The RNA samples were used as a template in qRT-PCR. The cDNA products were amplified in the presence of 3′ and 5′ primers (TNF-α, 5′-ATAGCTCCCAGAAAAGCAAGC-3′ and 5′-CACCCCGAAGTTCAGTAGACA-3′; iNOS, 5′-CTGCAGCACTTGGATCAGGAACCTG-3′ and 5′-GGGAGTAGCCTGTGTGCACCTGGAA-3′; *MIP-1α*, 5′-CCCAGCCAGGTGTCATTTTCC-3′ and 5′-GCATTCAGTTCCAGGTCAGTG-3′; MCP-1, 5′-GGAAAAATGGATCCACACCTTGC-3′ and 5′-TCTCTTCCTCCACCACCATGCAG-3′). A housekeeping gene, 18s (5′-TAAGTCCTGCCCTTTGTACACA-3′ and 5′-GATCCGAGGGCCTCACTAAAC-3′), was amplified in each experiment as control. The cycling condition steps for the amplification reaction consisted of a single 2-min heating step at 94 °C followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 1 min. The PCR-amplified products were electrophoresed and visualized on a 1% agarose gel. The intensities of the amplified products were analyzed using a StepOne Plus Real-Time PCR System (Applied Biosystems, USA). Data were analyzed according to the comparative threshold cycle (Ct) method, where the amount of target, normalized to an endogenous reference and relative to an experimental control.

2.13. Evaluation of TNF-α release from LPS-stimulated RAW264.7 cells

RAW264.7 cells were cultured overnight in a 96-well plate (2.5×10^5 cells/mL). Medium was removed before addition of fresh DMEM supplemented with 10% bovine serum. Secretion of TNF-α was monitored after treatment of RAW264.7 cells with the peptides at a concentration of 10 μg/mL for 30 min before treatment for 5 h at 37 °C with LPS (100 ng/mL). Samples of the medium from each treatment and untreated negative controls were then collected and the TNF-α concentration evaluated using a mouse TNF-α enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (ELISA, Boster). All experiments were done in triplicate.

2.14. Statistical analysis

All experiments were carried out on three independent occasions unless otherwise stated. All values are expressed as the mean ± standard error mean. Statistical comparisons were carried out by unpaired Student *t*-test or by analysis of variance (ANOVA) with SPSS 16.0.

3. Results

3.1. Peptide design and characterization

The structure and molecular weight of the peptides were verified by MALDI-TOF MS. Table 1 summarizes the theoretically calculated and measured molecular weights of each peptide. All peptides had measured molecular weight values in agreement with their theoretical values, suggesting that the peptides were successfully synthesized. The designed hybrid peptides showed the same mean hydrophobicity (−1.46) and increased hydrophobicity compared with protegrin-1 (−2.55). The relative hydrophobicity of hybrid peptides in aqueous solution indicated that differences in hydrophobicity were reliably reflected by different HPLC retention times. The retention times for LB-PG and CA-PG were 11.58 and 12.61 min, respectively, indicating the hydrophobic order: LB-PG < CA-PG.

The wheel-diagram and the relative hydrophobic moments showed that LB-PG (0.27) has a better balance between the hydrophobic and hydrophilic phases compared with cecropin A (0.22), LfcinB (0.24) and PG1 (0.23). In fact, all the hydrophilic amino acid residues of these peptides are located on one side, whereas the hydrophobic amino acid residues are on the other side of the helix in the helical wheel diagram (Fig. 1).

3.2. Secondary structure

It has been reported that PG-1 forms a β-hairpin structure in membrane-mimetic environments such as TFE, SDS micelles or liposome [25]. Accordingly, in this study, we investigated the second structure of the hybrid peptides. Fig. 3 shows the CD spectra of the parental and hybrid peptides at 0.1 mg/mL in DI water, 25 mM SDS and 50% TFE and Table 2 shows the calculated percentages of α-helix, β-hairpin, β-turn and random coil structures in parental and hybrid

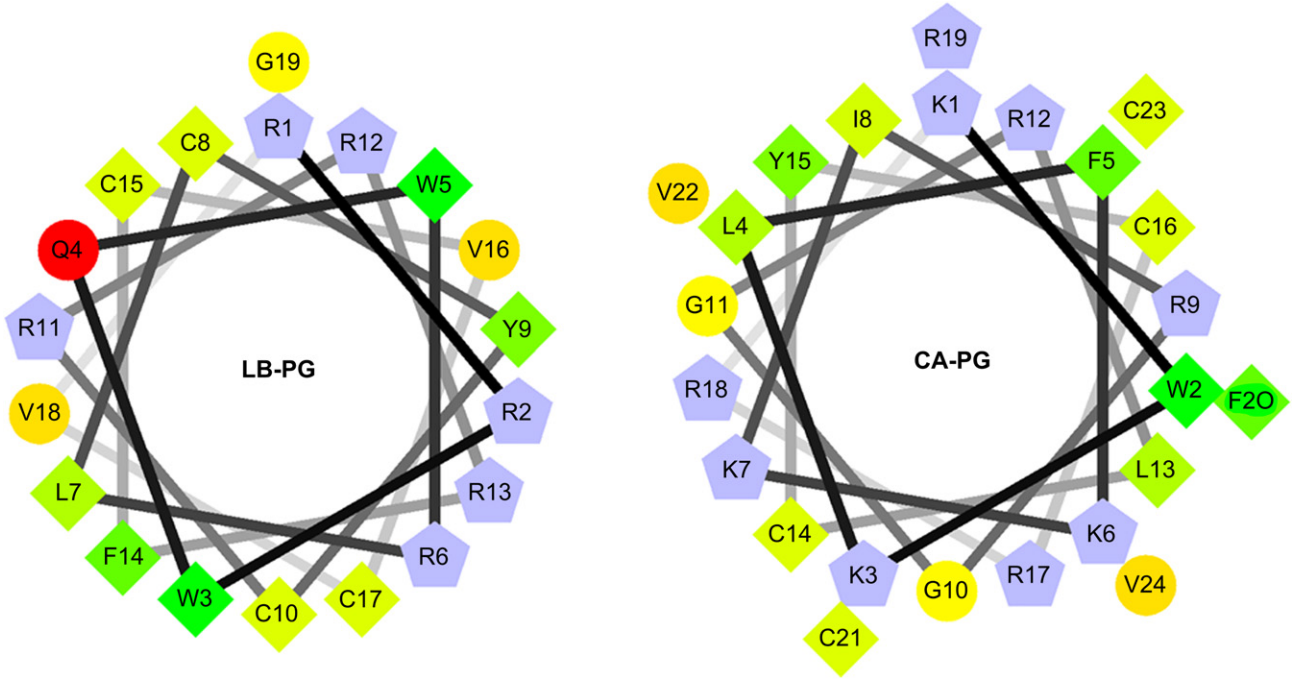

Fig. 1. Helical wheel projections of LB-PG and CA-PG. By default the output presents the hydrophilic residues as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is color coded as well: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. ('For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article').

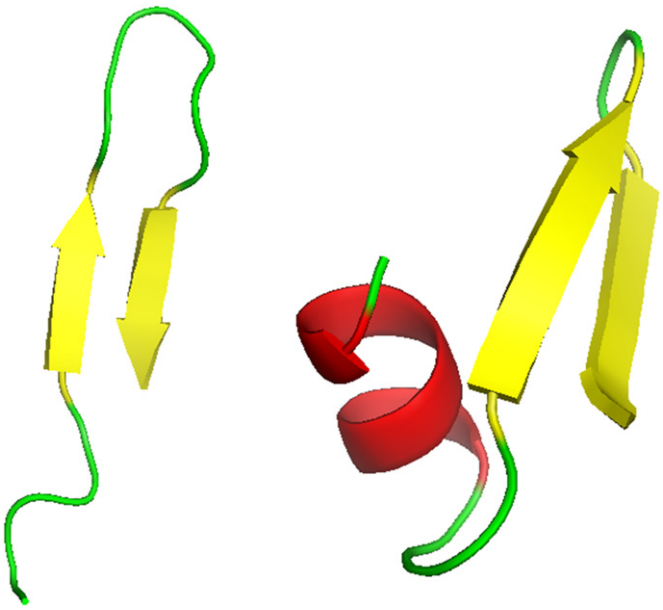

Fig. 2. Three-dimensional structure projections of LB-PG (left) and CA-PG (right).

peptides in the various solutions. As shown in Fig. 3, the CD spectrum indicated that parental and hybrid peptides had random coil conformations in DI water. Anionic surfactants such as SDS and fluorinated alcohols such as TFE are often used to mimic the cell membrane environment. CA exhibited well-defined positive and negative peaks at around 195 nm and two negative minimum bands at 208 and 222 nm in SDS and TFE, respectively, indicating predominance of α-helix structures (Fig. 3A). Fig. 3C and D show the CD spectra of LB-PG and CA-PG and the parental peptide PG-1 mixed into SDS and TFE in aqueous solution. In the membrane-like milieu, LB-PG and CA-PG adopted the same predominantly β-hairpin conformation as PG-1. However, the spectrum of CA-PG in SDS showed a substantial change, marked by intensification of both the negative peak around 200 nm and the peak around 225 nm, characteristic of a triple helix structure. Nevertheless, neither hybrid peptide exhibited a significant difference in β-hairpin content in a cell membrane mimicking environment such as TFE solution or SDS micelles (Table 2). We interpret these data to indicate that the β-hairpin structural characteristic of the peptide may play an important role in killing bacterial cells, but that its β-hairpin content may not be correlated to its antibacterial activities.

3.3. Antimicrobial activity

The antimicrobial activity of different concentrations of the peptides against Gram-negative strains and Gram-positive strains

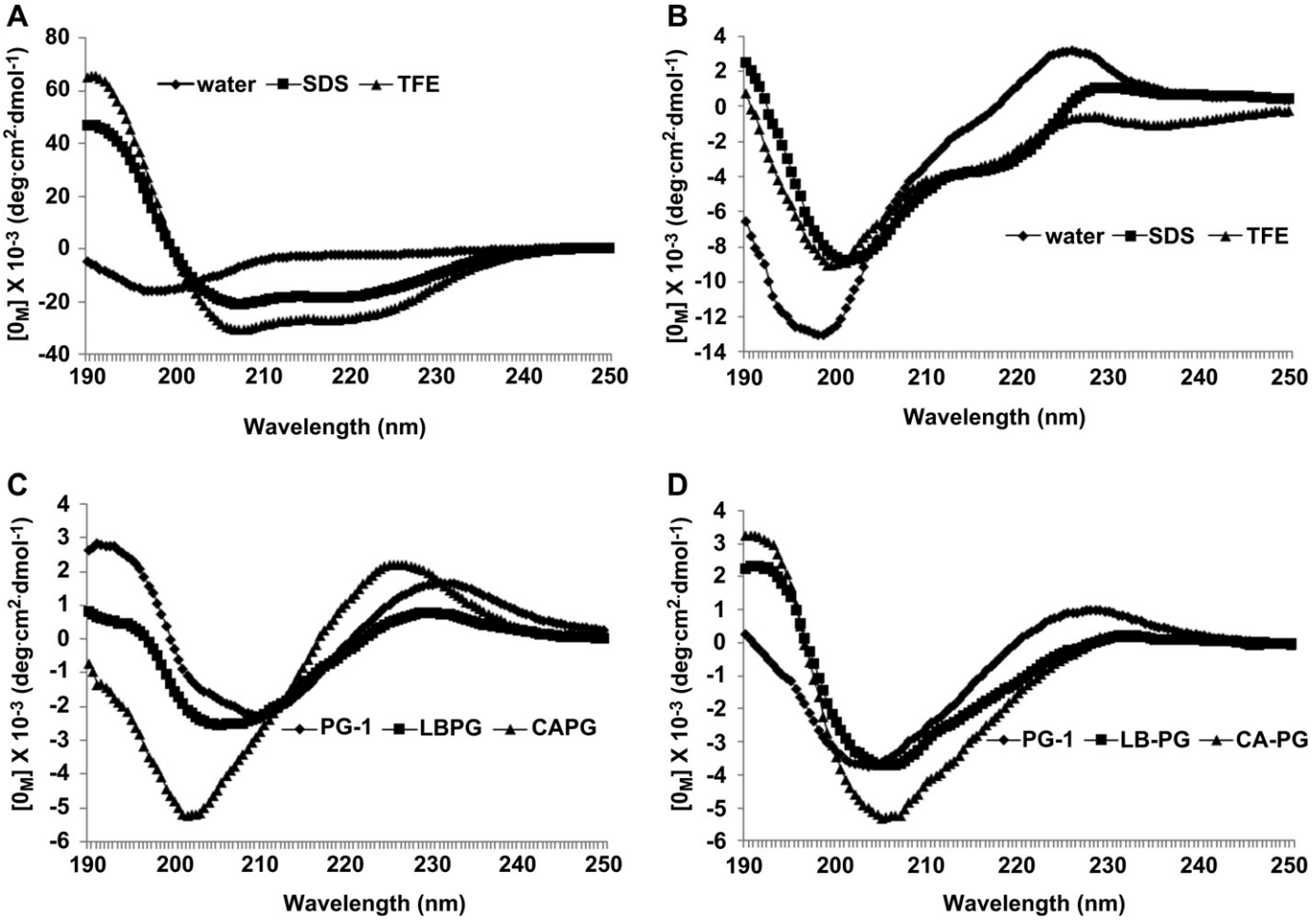

Fig. 3. The CD spectra of CA (A), LfcinB (B), PG-1, LB-PG and CA-PG in 25 mM SDS (C), and 50% TFE solution (D), the peptide concentrations are fixed at 0.1 mg/mL.

Table 2
Percentage of different secondary structure in parental and hybrid peptides in various solutions.

peptide	DI water			25 mM SDS			50%TFE		
	α-Helix	β-Sheet	Random	α-Helix	β-Sheet	Random	α-Helix	β-Sheet	Random
PG-1	0	69.1	30.9	7.5	76.4	16.1	0	64.4	35.6
LfcinB	0	40.0	60.0	0	53.2	46.8	0	48.5	51.5
CA	0	54.3	45.6	40.3	23.2	36.5	56.9	0	43.1
LB-PG	0	69.1	30.9	1.5	73.5	25.0	6.5	60.7	32.8
CA-PG	0	67.4	32.6	0	70.7	29.3	5.5	63.1	31.4

and beneficial bacteria is summarized in Table 3. The hybrid peptide LB-PG displayed antimicrobial activities similar to the natural peptide PG-1, and had approximately 2–8 fold higher antimicrobial activity than LfcinB, with an overall MIC of 8 μg/mL across the bacterial species. In comparison, the overall MIC of CA-PG was 32 μg/mL. While CA-PG did not maintain the same antibacterial activity as PG-1, it was higher than CA, and possessed an extended antibacterial spectra, especially against Gram-positive bacteria *S. aureus* and *S. epidermidis*. These assays confirmed that the hybrid AMPs have an extended range of activity in comparison to at least one of their parent peptides.

The inhibitory action of these peptides and antibiotics were also tested against intestinal commensal bacteria. Almost all peptides showed no activity against *L. acidophilus* and *B. suis*, while aureomycin and neomycin showed a marked inhibitory effect on *L. acidophilus* and *B. suis*.

3.4. Disruption to bacterial membrane integrity

Natural antibacterial peptides kill bacteria mainly via membrane permeabilization and subsequent structural disruption. SEM allowed us to directly observe cell morphology and integrity after peptide treatment. Based on their MIC and hemolytic values, the hybrid peptides appear to be more potent than the parental peptides. Hence, the microbicidal properties of these peptides were studied by observing the integrity of the bacterial cell membrane. As shown in Fig. 5, treatment with all peptides induced significant membrane damage in comparison to the control, which showed a bright and smooth surface without cellular debris. *E. coli* cells treated for 60 min with LB-PG and CA-PG at 1 × MIC became filamentous and elongated (Fig. 5E and F), and, similar to cells treated with LfcinB, did not appear to be dividing in a regular manner. Moreover, the membrane surface of peptide-treated *E. coli* cells showed more roughening and blebbing, as evinced by the formation of aggregates on the cell surface, and blebbing was more frequent in LB-PG-treated cells than in CA-PG-treated cells. In addition, treatment with CA-PG, which was not effective in inhibiting the growth of *E. coli*, resulted in minimal membrane damage in a small population of the bacteria. By comparison, the effects of peptides at 1 × MIC on *S. aureus* were shown in Fig. 6. Again, the morphological changes of the treated bacteria were obvious compared to the control (Fig. 6A). The cell surface became roughened and decorated with discrete blebs when treated with LB-PG and CA-PG. Moreover, LB-PG and CA-PG treated cells were damaged and became filamentous.

In addition to SEM, TEM was employed to study the morphology and intracellular alteration of bacteria. Fig. 7 shows that the integrity of the *E. coli* membrane upon the treatment with LB-PG and CA-PG was significantly adversely affected. AMP molecules disrupted the membrane of *E. coli*, changing the overall morphology of the bacterial cells, and some cytoplasmic clear zones could be seen. After 1 h of incubation with LB-PG, the *S. aureus* cell outer membranes appeared detached from the inner membrane, with a floral-like loop appearance (Fig. 8D). Leakage of intracellular contents was observed in the CA-PG-treated *S. aureus* cells, presumably due to the more severe loss of membrane integrity upon prolonged treatment of the bacteria.

Table 3
Antibacterial activity of parental and hybrid peptides.

	MIC (μg/mL)					Aureomycin	Neomycin
	PG-1	LfcinB	CA	LB-PG	CA-PG		
Gram-negative bacteria							
E. coli ATCC25922	8	32	16	8	32	4	2
E. coli K88	8	64	16	8	32	4	1
E. coli K12	8	32	32	8	16	2	2
E. coli EPEC O78:K88	32	128	64	16	32	64	1
S. choleraesuis CMCC50020	4	64	32	8	32	4	2
S. typhimurium CMCC50013	4	32	16	8	16	4	2
P. aeruginosa CMCC27853	8	128	32	16	32	2	4
Gram-positive bacteria							
S. aureus ATCC25923	2	16	> 256	8	32	0.06	0.13
S. epidermidis ATCC12228	4	16	256	8	32	0.13	0.5
Probiotic bacteria							
B. suis ATCC27533	128	> 256	> 256	> 256	> 256	32	4
L. acidophilus ATCC4356	> 256	> 256	> 256	> 256	> 256	8	64

3.5. DNA binding

Since there was no clear evidence that the mechanism of action of the peptides was restricted to membrane permeabilization, we investigated the possibility of the intracellular effects, such as the

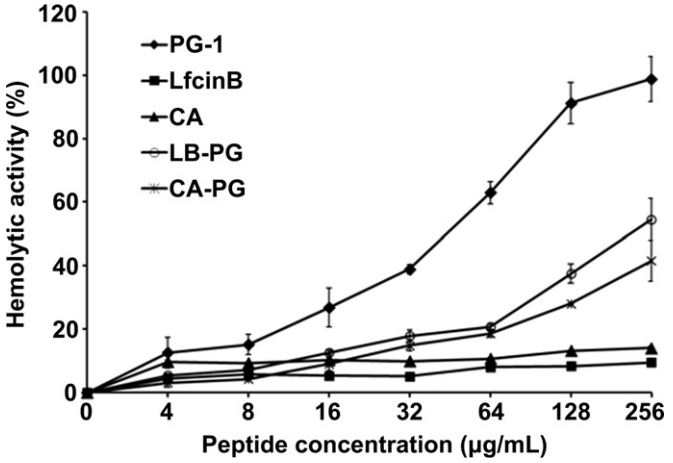

Fig. 4. Concentration-response curves of the peptides against porcine red blood cells. The hemoglobin release was monitored with a Microplate autoreader by measuring the absorbance at 576 nm.

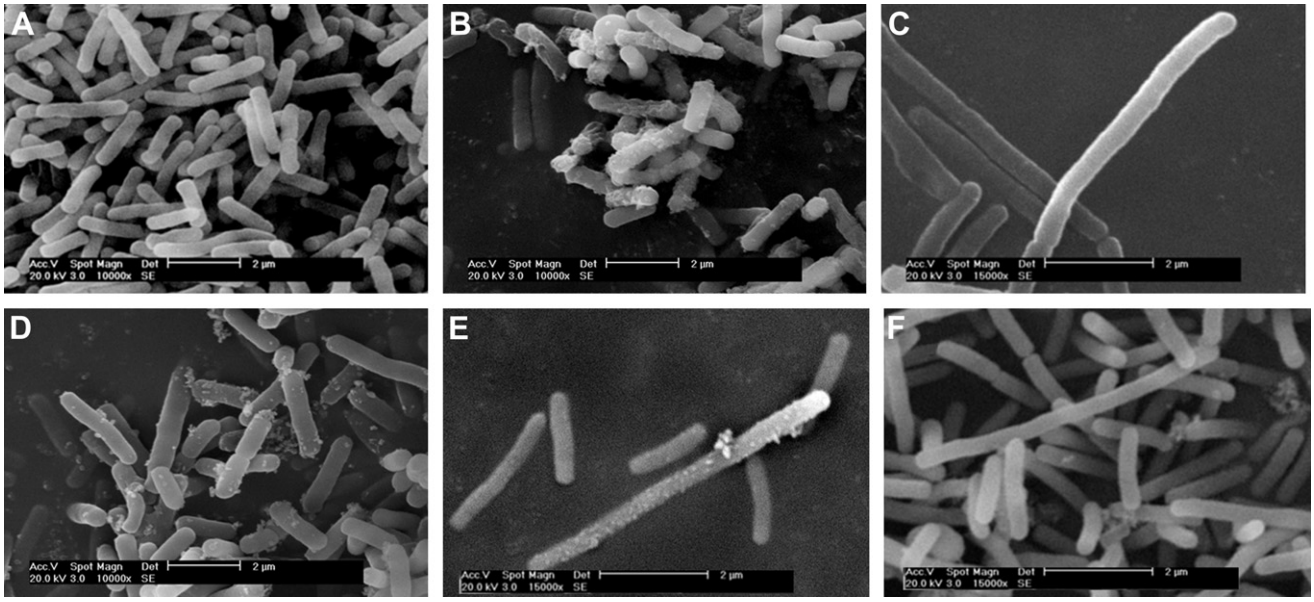

Fig. 5. SEM micrographs of *E. coli* ATCC25922: (A) Control; (B) PG1-treated; (C) LfcinB-treated; (D) CA-treated; (E) LB-PG-treated, and (F) CA-PG-treated. Bacteria were treated with peptides at 1 × MIC for 1 h. The control was done without peptides. Scale bar = 2 μm.

DNA-binding properties of peptides. As shown in Fig. 9, LB-PG inhibited DNA migration above a weight ratio of 1.0. Similar patterns of migration were observed with the parental peptides LfcinB and PG-1. In contrast, CA-PG did inhibited DNA migration only above a ratio of 8.0, which was possibly due to a lack of intracellular targets of CA (data not shown).

3.6. Synergy with conventional antibiotics

One of the known assets of antimicrobial peptides is their potential to promote the activity of conventional antibiotics. Not all peptides possess this activity however, and the structural basis for the synergism has not been investigated. The FICI data for combinations of hybrid peptides are summarized in Table 4. CA-PG exhibited synergistic effects with both antibiotics against *E. coli* and *S. aureus* cells, and in both cases, FIC indices from 0.375 to 0.5 were achieved. Although there were no similar synergistic effects between LB-PG and the antibiotics, the MICs of the antibiotics decreased noticeably upon addition of the peptides (data not shown), indicating that LB-PG and CA-PG may have potential as feed additives to reduce the dietary dose of antibiotics.

3.7. Hemolytic activity

The toxicity of hybrid peptides against eukaryotic cells was determined as the ability to lyse porcine erythrocytes measured at

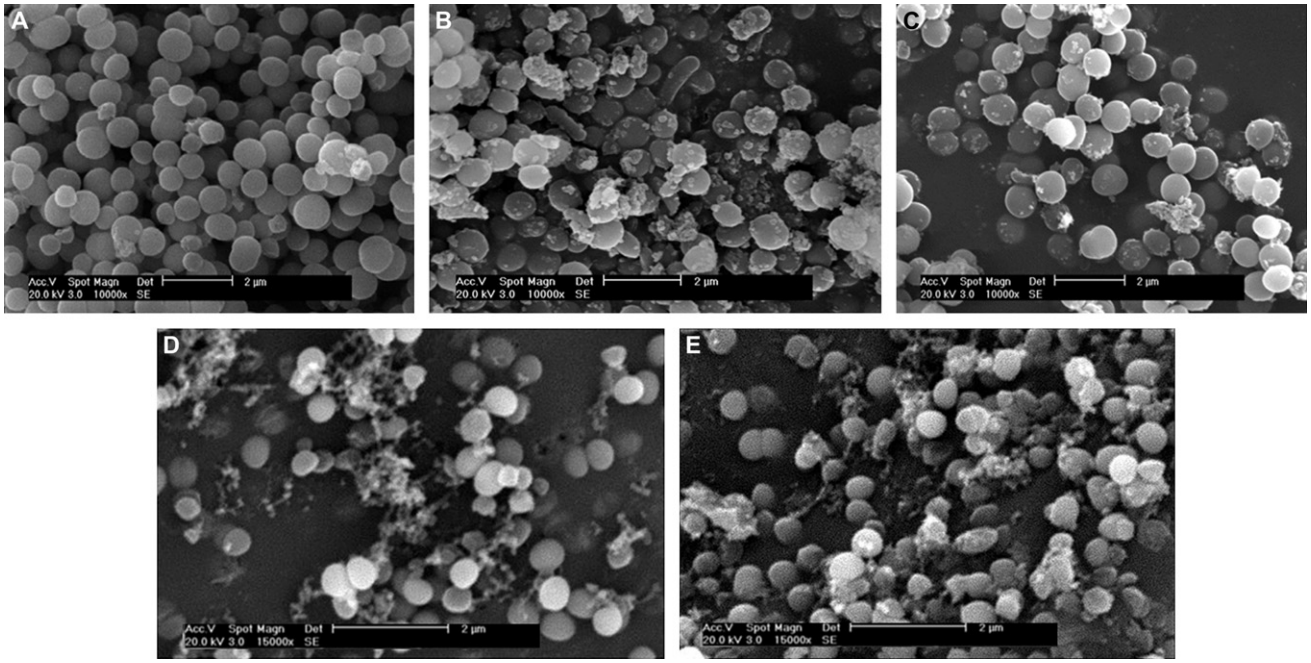

Fig. 6. SEM micrographs of *S. aureus* ATCC25923: (A) Control; (B) PG1-treated; (C) LfcinB-treated; (D) LB-PG-treated, and (E) CA-PG-treated. Bacteria were treated with peptides at 1 × MIC for 1 h. The control was done without peptides. Scale bar = 2 μm.

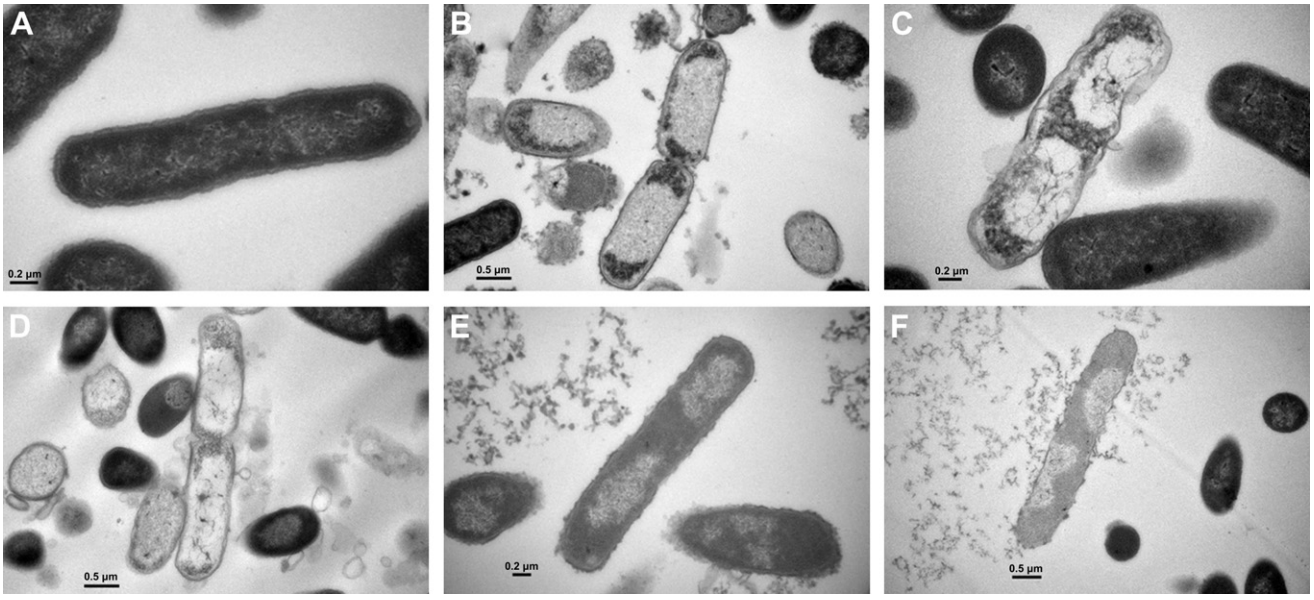

Fig. 7. TEM micrographs of *E. coli* ATCC25922: (A) Control; (B) PG1-treated; (C) LfcinB-treated; (D) CA-treated; (E) LB-PG-treated, and (F) CA-PG-treated. Bacteria were treated with peptides at 1 × MIC for 1 h. The control was done without peptides. Scale bar = 0.2 μm.

serial peptide concentrations alongside their parental peptides (Fig. 4). LfcinB and CA showed the lowest hemolytic activity among these peptides, namely, 9.57% and 14.10% respectively, even at the highest concentration of 256 μg/mL. In contrast, PG-1 displayed the maximal hemolytic activity, with almost 99% lysis, at a concentration of 256 μg/mL. The hybrid peptides LB-PG and CA-PG demonstrated relatively low hemolytic activity, showing a 2–4 fold decrease at each concentration when compared to PG-1. However, they were more hemolytic than the parental peptides LfcinB and CA.

3.8. Resistance to proteolytic digestion

A major limitation of antimicrobial peptides is their inactivation by proteases, and poor protease stability severely limits the practical application of many peptides as feed additives. Using an antimicrobial assay with *E. coli* ATCC25922, we therefore examined the susceptibility of peptides to pepsin and trypsin. As shown in Fig. 10(A), pepsin treatment of CA completely abolished antimicrobial activity against *E. coli*, while pepsin treatment of CA-PG partially preserved its antimicrobial activity. In contrast, the antimicrobial activities of hybrid peptide LB-PG and parental peptides PG-1 and LfcinB were completely preserved after pepsin treatment. The same trend was observed after treatment with trypsin (Fig. 10B), although the inhibitory effect of all these peptides was partially abolished in this case. Next the disappearance of the intact peptides incubated in SGF and SIF was followed by RP-HPLC. LB-PG seems to confer some stability against parental peptides in SGF, as it has half-lives of about 10 min (Fig. 11A). While other peptides are degraded quickly with half-lives of less than 5 min.

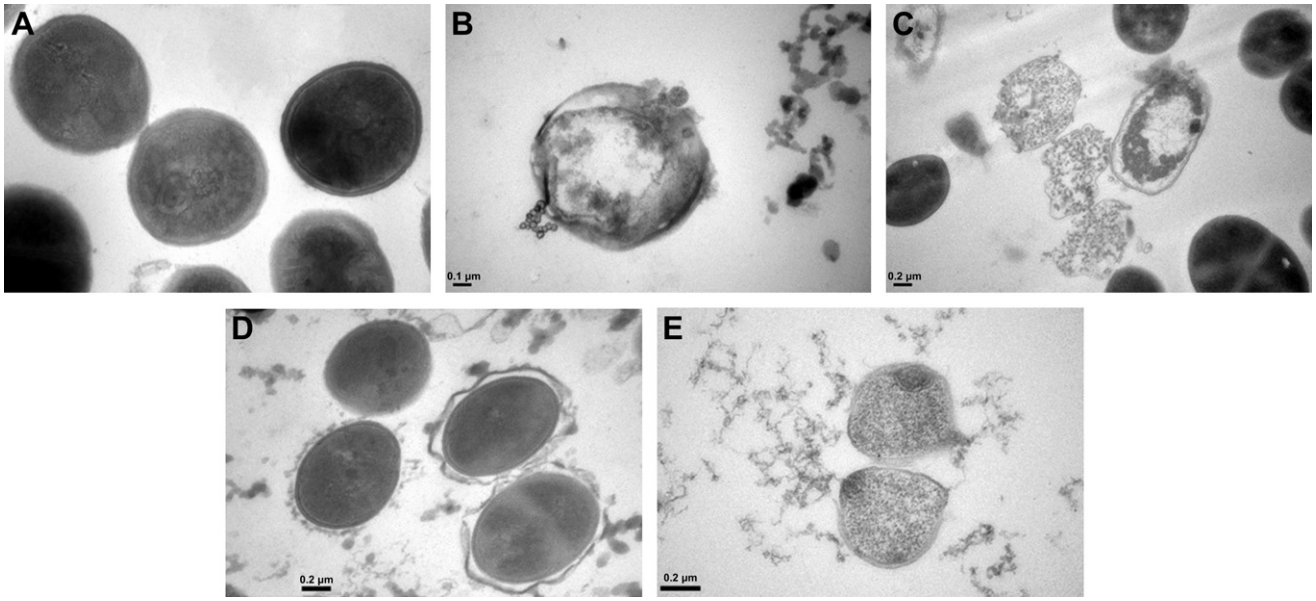

Fig. 8. TEM micrographs of *S. aureus* ATCC25923: (A) Control; (B) PG1-treated; (C) LfcinB-treated; (D) LB-PG-treated, and (E) CA-PG-treated. Bacteria were treated with peptides at 1 × MIC for 1 h. The control was done without peptides. Scale bar = 0.2 μm.

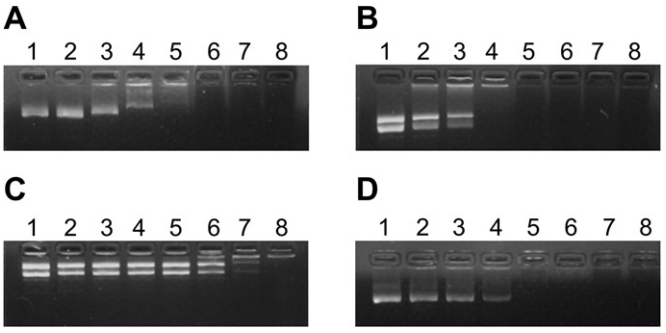

Fig. 9. Interaction of peptides with plasmid DNA. Binding was assayed by measuring inhibition of migration by plasmid DNA. Various amounts of peptides were incubated with 400 ng for 1 h at room temperature prior to electrophoresis on a 1.0% agarose gel. A. LfcinB; B. PG-1; C. CA-PG; D. LB-PG. The weight ratio (peptide:DNA) is indicated as follows: lane 1, plasmid DNA alone; lane2, ratio 1:4; lane3, ratio 1:2; lane 4, ratio 1:1; lane 5, ratio 2:1; lane 6, ratio 4:1; lane7, ratio 8:1; lane 8, ratio 16:1.

3.9. Cytotoxicity on RAW264.7 macrophage cells

The effect of the peptides on proliferation of RAW264.7 macrophage cells was evaluated by WST-1 assay and results are shown in Fig. 12A. In all cases, LfcinB and CA exhibited the lowest inhibition, while PG-1 showed the highest inhibition. No peptides exhibited significant cytotoxicity between 2 and 128 μg/mL, which was also observed in the case of the hybrid peptides LB-PG and CA-PG.

A dose-dependent increase in extracellular LDH was observed with all peptides (Fig. 12B). Among all the peptides, PG-1 caused a loss of plasma membrane integrity. In contrast, treatment of RAW264.7 cells with hybrid peptides LB-PG and CA-PG, did not significantly induce LDH release, indicating that LB-PG and CA-PG maintain the integrity of the plasma membrane in RAW264.7 cells. Peptides exhibiting no cytotoxicity at 10 μg/mL were used in the experiments described below.

3.10. Inhibition of LPS-mediated cytokines expression

To test whether hybrid peptides could inhibit the LPS-induced proinflammatory response, the murine macrophage cell line RAW264.7 was used as a model. qRT-PCR results, shown in Fig. 13, show that all hybrid peptides and parental peptides were potent inhibitors. Quantitative analysis showed that LB-PG and CA-PG, applied at 10 μg/mL in the presence of LPS, caused a remarkable decrease in mRNA expression of TNF-α (Fig. 13A). The same trend also occurred with iNOS, *MIP-1α* and MCP-1 (Fig. 13B, C and D). Collectively, these data strongly support the potential of LB-PG and CA-PG as both antibacterial and anti-inflammatory agents.

3.11. Inhibition of TNF-alpha release from LPS-stimulated RAW264.7 cells

We next used a TNF-α enzyme-linked immunosorbent assay kit to evaluate TNF-α concentration in the cell medium after LPS (100 ng/mL) stimulation in the presence of the hybrid peptides. Fig. 14 shows that, consistent with the qRT-PCR result, all peptides attenuated the level of TNF-α secretion. LB-PG, the most active peptide, reduced TNF-α secretion to basal level.

Table 4
The FIC indexes[a] for hybrid peptides LB-PG, CA-PG in combination with aureomycin and neomycin against *E. coli* ATCC25922 and *S. aureus* ATCC25923.

Strain	LB-PG + aureomycin	CA-PG + aureomycin	LB-PG + neomycin	CA-PG + neomycin
E. coli ATCC25922	0.75	0.375	1.5	0.375
S. aureus ATCC25923	1.25	0.5	2.0	0.5

[a] Synergic action is defined as FICI $\leq$ 0.5, no interaction as 0.5 < FICI $\leq$ 4 and antagonistic action as FICI > 4.

4. Discussion

AMPs represent a class of antibiotic agents that may be useful in the treatment of a range of infectious diseases, some of which have also been found to exert immunoregulatory effects [26]. Most AMPs kill bacteria through targeting and disrupting the integrity of plasma membranes, and such a unique mode of action significantly reduces the occurrence of bacterial resistance [27]. The use of synthetic biomaterials, however, often leads to interactions between them and the host, inducing ectopic cytotoxic and immunogenic responses and posing potential threats to public health [28,29]. To circumvent these inherent limitations, a number of technological hurdles must be addressed, including optimizing peptide stability and antimicrobial activity, reducing high hemolytic activity, and creating peptidomimetics which recapitulate the critical biophysical properties of antibacterial peptides such as positive charge and amphiphilicity. Deficits exist however in the rational design of peptide structures, as well as in correlations between such information and their performance. It is thought that by promoting their incorporation into membranes, an amphipathic secondary structure is especially crucial for antimicrobial activity [30]. An overall positive charge favors their binding to negatively charged membranes through electrostatic attraction and thereby imposes selectivity [31]. The molecular hydrophobicity reflects the intrinsic ability of a peptide to move from aqueous into hydrophobic phase [32]. While these structural parameters have not been stringently correlated to antimicrobial activity, an appropriate balance among them is required to maintain desirable activities while removing undesirable properties [33].

Hybridization is an effective strategy for the design of novel antimicrobial peptides [17], since hybrid peptides ideally possess a more desirable combination of various parameters than their parental peptides. Previous studies focused on designing hybrid peptides which form α-helices, which is the major structure of AMPs [34–36]. Others have created novel hybrid AMPs with the aim of increasing antibacterial activity or reducing the hemolytic activity of the parental peptides [37]. It is of interest to note that of all the hybrid peptides designed, cecropin A has been the peptide most frequently used as the parental peptide, possibly due to its high α-helical conformation. Jin *et al.* reported that antimicrobial peptides that can form amphipathic β-hairpin conformations may offer a selective advantage over α-helical peptides in targeting bacterial cells [30].

In the present study therefore, two hybrid β-hairpin peptides termed LB-PG (LfcinB residues 4–9 followed by PG-1 residues 5–17), and CA-PG (cecropin A residues 1–8 followed by PG-1 residues 1–16) were designed and studied. While both hybrid peptides are inherently amphiphilic and adopt amphiphilic structures, LB-PG has a higher potential to form amphiphilic structure than CA-PG (Table 1). Most previously reported natural amphiphilic antimicrobial peptides adopt flexible random structure in aqueous solutions and assemble into a more rigid α-helical structure in the presence of a simulated membrane environment. In order to investigate the proclivity of the conformational structures of the hybrid peptides in both water and membrane-like environments, they were first tested with CD spectroscopy technique at room temperature, by using both water and the membrane-like SDS micelle and TFE solutions as the solvents (Fig. 3 and Table 2). Our results indicated that LB-PG and CA-PG have higher β-hairpin probability than the parental peptide PG-1, which was in line with

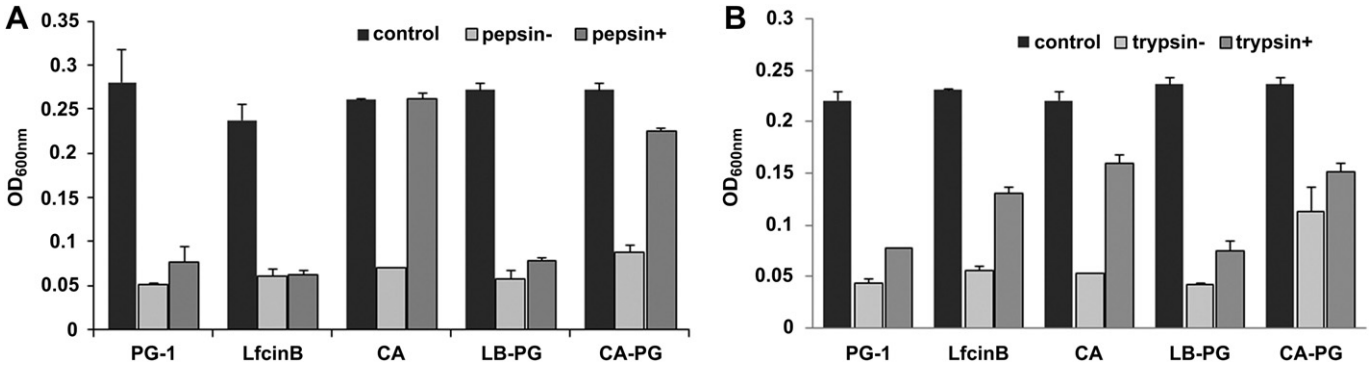

Fig. 10. Inhibition of antimicrobial activity of the peptides against *E. coli* ATCC25922 by pepsin (a) and trypsin (b) assessed using the broth microdilution method.

the prediction by I-TASSER (Fig. 2). Consequently, LB-PG and CA-PG can be classified as β-hairpin cationic amphipathic peptides.

The antimicrobial assays demonstrated that LB-PG and CA-PG possessed remarkable antimicrobial activity and broader antimicrobial spectra when compared to at least one parental peptide (Table 3). This result is most likely due to an increase in the net positive charge, amphipathicity, and β-hairpin propensity. As observed in previous reports, an increase in cationic charge favors the initial electrostatic attraction of peptides to the negatively charged membranes of bacteria [31]. In this study, CA-PG (+9) has higher net charge than LB-PG (+6), and while we would accordingly expect enhanced antibacterial activity for CA-PG, this was not observed − rather, LB-PG had more effective bactericidal capacity. CA-PG did not display any improvement in antibacterial activity over PG-1, although it had a higher antibacterial activity than CA. The retention time of peptides on a reverse-phase matrix has been reported to be related to peptide hydrophobicity [38]. With an increase in retention time, the molecular hydrophobicity of CA-PG was also enhanced. There is no simple correlation with antibacterial effect however. For example, although CA-PG is the most hydrophobic molecule (retention time = 12.61 min), its antibacterial activity is weaker than LB-PG. This situation was similar to the phenomenon reported by Chen et al. for α-helix peptides [37] and for the lipopeptides designed by Laverty et al. [39]. In both cases, there was a threshold of hydrophobicity related to peptide membrane disrupting power: an initial increase in hydrophobicity enhances the antimicrobial activity as in the case of LB-PG, but a further increase in hydrophobicity, as in the case of CA-PG, makes the molecules too hydrophobic to retain the antibacterial activity. It is likely that in our case amphipathicity may be the most important factor in the design of hybrid peptides. LB-PG has a higher potential to form amphiphilic structure than CA-PG, which is in agreement with its ability to inhibit the growth of bacteria. Increasing amphipathicity would result in increasing antibacterial capacity because the charge increase might facilitate stronger peptide binding to the negatively charged membrane surface of bacteria. The length of the designed peptides should also be taken into consideration, although this property is not as important as those discussed above.

The peptides designed in this study were aimed to fight against infectious disease with reduced hemolysis. Since hemolysis of erythrocytes is often thought to be one of the bottlenecks for the application of AMPs, it was important to evaluate the cytotoxic effect of the peptides. The hemolytic assays showed that both LB-PG and CA-PG had lower hemolytic activity than the parental peptide PG-1, but higher activity than LfcinB and CA (Fig. 4). At the MIC levels for most bacteria tested, LB-PG and CA-PG induced 7.17% and 14.84% hemolysis, respectively, reflecting good selectivity of these peptides towards the anionic constituent of microbial cell membranes over the zwitterionic mammalian cell membranes.

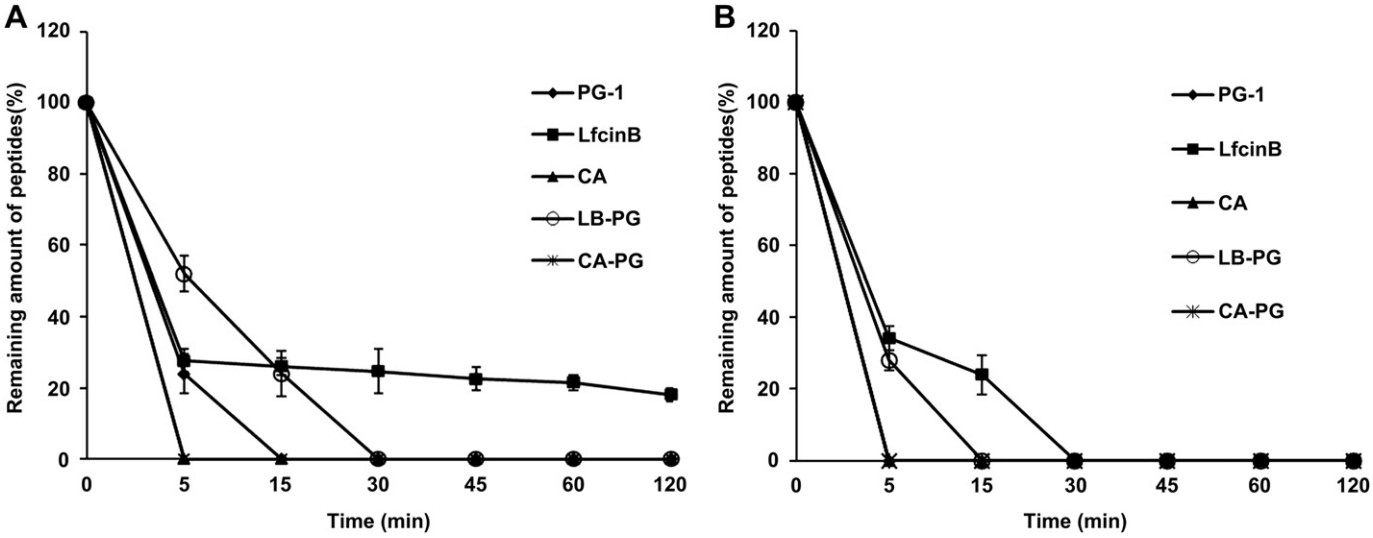

Fig. 11. Degradation of peptides in SGF (A) and SIF (B). The peptides were incubated with the SGF or SIF for different time periods and the remaining peptide amounts were determined by RP-HPLC.

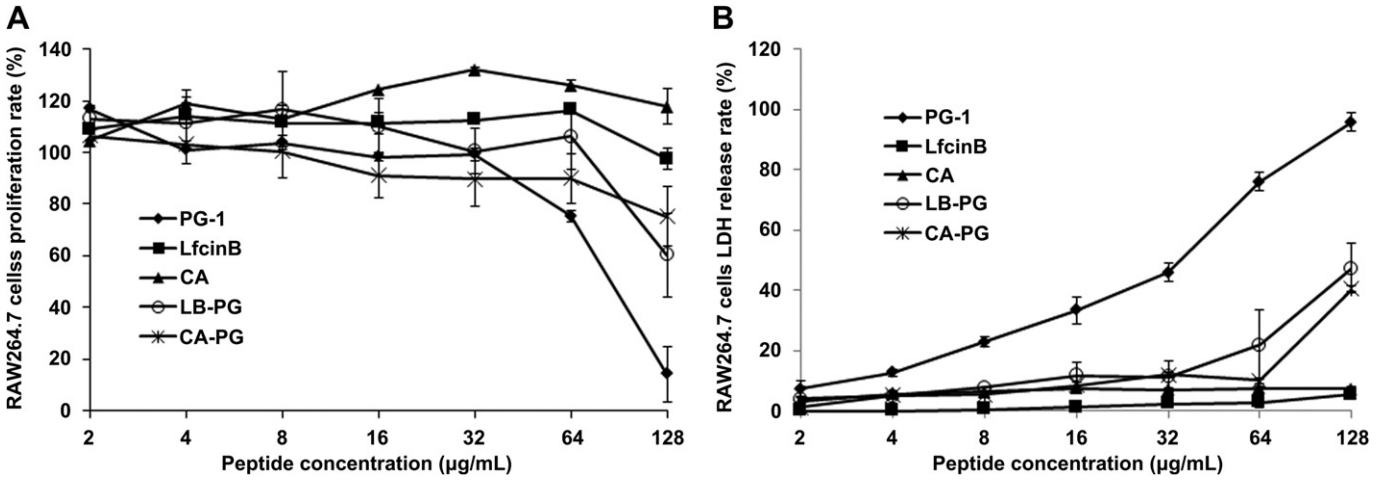

Fig. 12. Cell viability (A) and LDH release (B) of RAW264.7 cells in the absence or presence of hybrid peptides and parental peptides.

Presumably, this improved selectivity was due to the increased hydrophobicity and better amphiphilicity of these peptides. While it is well known that higher hydrophobicity often correlates with more hemolytic activity, our study indicates that this may not be the case for β-hairpin AMPs. Low hemolytic activity at relatively high peptide concentrations, together with modified antibacterial activity, is an attractive combination of properties for many applications of these peptides in the development of novel antibiotics. It is worth noting that hemolytic activity often correlates with improved antimicrobial activity due to similar determinants such as helical probability and hydrophobicity. In this context, it is not surprising that CA-PG seemed to possess greater ability to

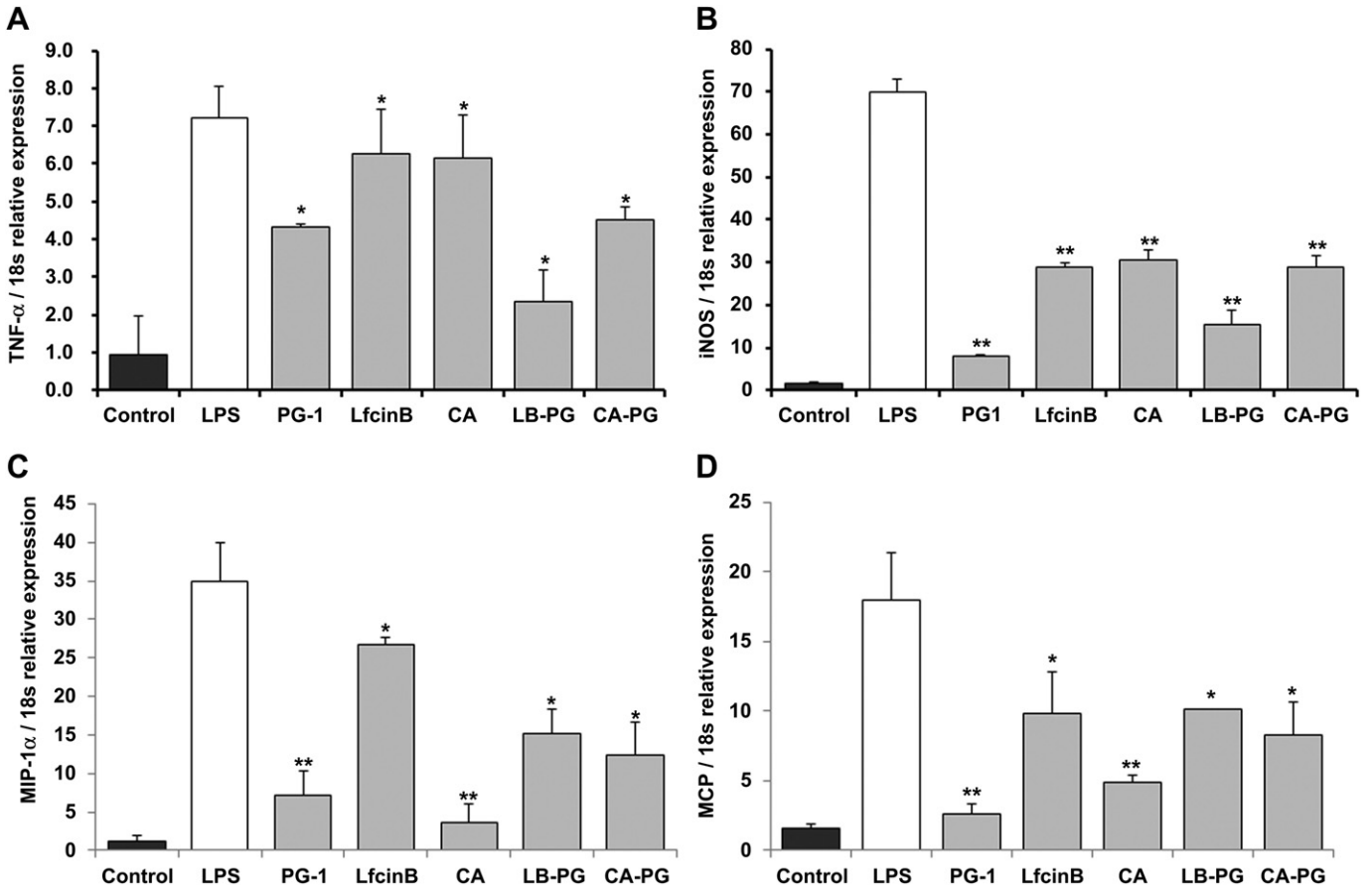

Fig. 13. Neutralization of LPS by hybrid peptides and parental peptides. A, blockage of LPS-induced TNF-α gene expression by AMPs. B, blockage of LPS-induced iNOS gene expression by AMPs. C, blockage of LPS-induced MIC-1α gene expression by AMPs. D, blockage of LPS-induced MCP-1 gene expression by AMPs. RAW264.7 cells were pretreated for 30 min with 10 μg/mL peptides and then stimulated with 100 ng/mL LPS or left untreated for 5 h. Data shown are representative of three independent experiments with similar results.

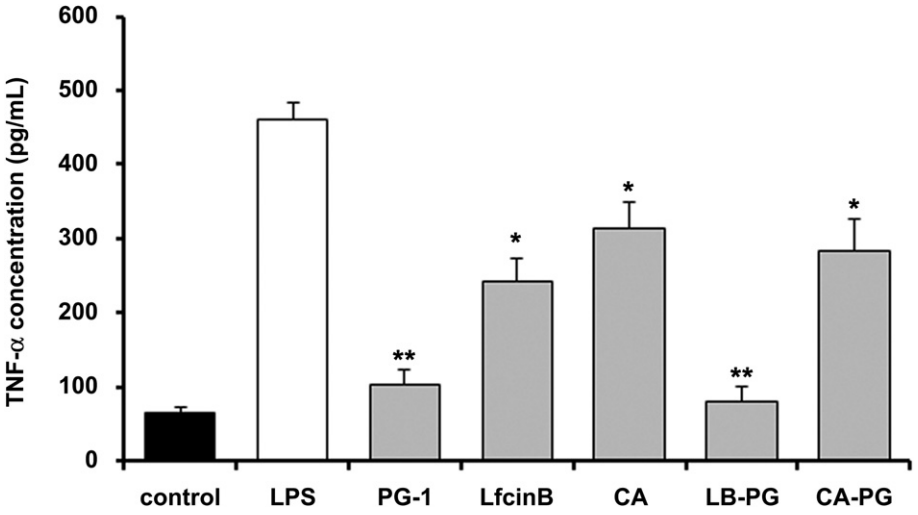

Fig. 14. Effects of the peptides on LPS-induced TNF-α secretion by macrophages. Cells were stimulated with LPS (100 ng/mL) in the presence or absence of peptides. After incubation, the TNF-α concentration in the cell medium was evaluated using a mouse TNF-α ELISA kit.

penetrate and/or disrupt the cell membrane due to the increased hydrophobicity of the longer peptide molecule, hence exhibiting an increased hemolytic activity. In our case, CA-PG exhibited lower hemolytic activity than LB-PG. An optimal balance therefore exists between hydrophobicity and amphiphilicity when designing peptides.

Most AMPs have cationic and amphipathic properties which disrupt membranes or pore/ion channel formation in the cytoplasmic membranes of susceptible bacteria [40]. Other mechanisms of action have been reported, including inhibition of cellular functions by binding to DNA, RNA, and proteins, thereby inhibiting DNA, RNA, and/or protein synthesis [41]. Our SEM and TEM studies confirmed that these amphiphilic β-hairpin hybrid peptides kill bacteria via the same action (membrane disruption) as that of their parental peptide PG-1. LB-PG treated bacteria displayed disrupted membranes with the appearance of some holes. CA-PG showed similar membrane disturbance, but to a lesser extent, consistent with its different microbicidal capacity. Elongation of bacteria suggested that other bactericidal target of hybrid peptides might be present inside the cell. LB-PG inhibited the migration of DNA on the gel at a ratio of 2:1, suggesting it may have a role in interfering with bacterial DNA synthesis. In contrast, CA-PG bound DNA only at much higher concentrations, indicating that similar to the parental peptide CA, membrane disruption is its primary mode of action.

It has recently been shown that synergistic effects among antibiotic agents could improve the antibacterial effect, especially in the case of drug-resistant bacteria [42]. This is in turn may decrease the side effects of antibiotics in applications such as residues in animal products, and inducement of drug-resistant bacteria. Little is known regarding synergistic effects between AMPs and animal feed additives however. Our data indicate that the hybrid peptide CA-PG exhibited synergistic action with aureomycin and neomycin against *E. coli* ATCC25922 and *S. aureus* ATCC25923 (Table 4). Although there was no synergistic effect between LB-PG and antibiotics towards the two strains, MICs of aureomycin were 12–50% of those when used individually, suggesting that in combination, CA-PG and LB-PG could potentially be used to decrease the dose of antibiotics.

The suppression of antimicrobial peptide activity by proteolytic digestion has hampered efforts to develop safe and effective peptides for clinical use. In our study, LB-PG and CA-PG retained, respectively, complete and partial antimicrobial activity after pepsin and trypsin treatment, indicating that these peptides are candidates for clinical therapeutic use. It is possible that the inactivity of CA-PG may be due to proteolytic susceptibility, a phenomenon that has previously been reported for other AMPs exposed to trypsin [43]. Cylization can be a viable strategy to constrain the peptide structure and thereby enhance antimicrobial activity [44]. This approach has been used for an analogue of indolicidin, where a disulfide-bridged peptide was more resistant to trypsin degradation than its linear counterpart [45]. We assume that the disulfide is essential for stabilizing the β-sheet structure of LB-PG, as it appears 69.2% β-sheet structure in aqueous solution.

Another undesirable property of biomaterials stems from immunogenicity arising from the interaction of AMPs and the host organism via Toll-like Receptor (TLR) pathways, which occurs in a medical condition referred to as hypercytokinemia or cytokine storm, and which causes further severe inflammation [46]. LPS released from Gram-negative bacteria is one of the most powerful stimulants of the immune system, and activates TLR4 on macrophages, monocytes, and neutrophils, which then release prototypic pro-inflammatory cytokines (e.g., TNF, iNOS) and chemotaxin (e.g., *MIP-1α* and MCP-1) [47,48]. Certain AMPs, including LL-37 [49], indolicidin [50] and defensin [51], can suppress cytokine production by LPS-stimulated macrophage cells. A combination of antimicrobial and anti-inflammatory properties makes AMPs attractive candidates in applications such as feed additives in diets. In particular, LB-PG and CA-PG exhibited good anti-inflammatory activity at concentrations which were not associated with cytotoxicity. These peptides significantly inhibited TNF-α production and gene expression in LPS-stimulated RAW264.7 cells at a concentration of 10 μg/mL. In addition, iNOS is characterized as a key mediator of inflammation, which might be responsible for oxidative/nitrosative tissue injury [52]. In this study, LB-PG and CA-PG significantly reduced iNOS mRNA expression compared with LPS-stimulated RAW264.7 cells. Similarly, preincubation with LB-PG and CA-PG reduced the release of chemokines, a phenomenon reported to play important roles in a wide variety of diseases by inducing accumulation and activation of monocytes, and which may contribute to the lethal outcome of sepsis [53]. *MIP-1α* is produced by monocytes, neutrophils, activated lymphocytes, and fibroblasts and is chemotactic for macrophages and T lymphocytes [54]. Moreover, it has been shown to be critically involved in the TNF-α-induced recruitment of leukocytes [55].

5. Conclusion

In this study, we have highlighted that design of AMPs via the hybridization of different AMPs significantly broadens their antimicrobial activity against Gram-positive and Gram-negative bacteria, and reduces their hemolytic activity. This excellent selectivity was due particularly to a combination of proper hydrophobic-cationic balance, stronger inclination to form β-hairpin conformation in a membrane-like environment, and an optimum facially amphiphilic structure conformation. The antimicrobial mechanism of this peptide is shown not only by pore formation and membrane damage, but also by intracellular DNA-binding. The hybrid peptides significantly inhibited the expression and production of pro-inflammatory cytokines (TNF-α and iNOS) and chemokines (*MIP-1α* and MCP-1) in LPS-stimulated mouse macrophage RAW264.7 cells. The findings reported in this study provide a strong rationale for further developing these AMPs as active anti-infective agents for treating drug resistant infection caused by various microbes. Although the results reported here reflect the in vitro situation and are at an early stage, the rationalization of peptide design and optimization will be useful during future assessments of in vitro and in vivo models, with the ultimate aim of exploiting their antibacterial potential in technological applications such as biomedical coatings, cosmetic and healthcare formula.

Acknowledgments

We gratefully acknowledge financial support from the National Science Fund for Distinguished Young Scholars of China (Grant No. 31025027) and the earmarked fund for Modern Agro-industry Technology Research System (CARS-36)

References

[1] Auvynet C, Rosenstein Y. Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity. FEBS J 2009;276:6497–508.
[2] Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biothechnol 2006;24:1551–7.
[3] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;24:389–95.
[4] Hancock REW. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1:156–64.
[5] Shai Y. Mode of action of membrane active antimicrobial peptides. Biopolymers 2002;66:236–48.
[6] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55:27–55.
[7] Navon-Venezia S, Feder R, Gaidukov L, Carmeli Y, Mor A. Antibacterial properties of dermaseptin S4 derivatives with in vivo activity. Antimicrob Agents Chemother 2002;46:689–94.
[8] Bell A. Antimalarial peptides: the long and the short of it. Curr Pharm Des 2011;17:2719–31.
[9] Shukla A, Fleming KE, Chuang HF, Chau TM, Loose CR, Stephanopoulos GN, et al. Controlling the release of peptide antimicrobial agents from surfaces. Biomaterials 2010;31:2348–57.
[10] Wiradharma N, Khan M, Yong LK, Hauser CA, Seow SV, Zhang S, et al. The effect of thiol functional group incorporation into cationic helical peptides on antimicrobial activities and spectra. Biomaterials 2011;32:9100–8.
[11] Chen C, Hu J, Zhang S, Zhou P, Zhao X, Xu H, et al. Molecular mechanisms of antibacterial and antitumor actions of designed surfactant-like peptides. Biomaterials 2012;33:592–603.
[12] Wiradharma N, Khoe U, Hauser CA, Seow SV, Zhang S, Yang YY. Synthetic cationic amphiphilic α-helical peptides as antimicrobial agents. Biomaterials 2011;32:2204–12.
[13] Hilpert K, Elliott MR, Volkmer-Engert R, Henklein P, Donini O, Zhou Q, et al. Sequence requirements and an optimization strategy for short antimicrobial peptides. Chem Biol 2006;13:1101–7.
[14] Chou HT, Kuo TY, Chiang JC, Pei MJ, Yang WT, Yu HC, et al. Design and synthesis of cationic antimicrobial peptides with improved activity and selectivity against *Vibrio spp*. Int J Antimicrob Agents 2008;32:130–8.
[15] Blondelle SE, Lohner K. Optimization and high-throughput screening of antimicrobial peptides. Curr Pharm Des 2010;16:3204–11.
[16] Park KH, Nan YH, Park Y, Kim JI, Park IS, Hahm KS, et al. Cell specificity, anti-inflammatory activity, and plausible bactericidal mechanism of designed Trp-rich model antimicrobial peptides. Biochim Biophys Acta 2009;1788:1193–203.
[17] Saugar JM, Rodríguez-Hernández MJ, de la Torre BG, Pachón-Ibañez ME, Fernández-Reyes M, Andreu D, et al. Activity of cecropin A-melittin hybrid peptides against colistin-resistant clinical strains of Acinetobacter baumannii: molecular basis for the differential mechanisms of action. Antimicrob Agents Chemother 2006;50:1251–6.
[18] Liu YF, Luan C, Xia X, An S, Wang YZ. Antibacterial activity, cytotoxicity and mechanisms of action of cathelicidin peptides against enteric pathogens in weaning piglets. Int J Pept Res Ther 2011;17:175–84.
[19] Liu YF, Han FF, Xie YG, Wang YZ. Comparative antimicrobial activity and mechanism of action of bovine lactoferricin-derived synthetic peptides. Biometals 2011;24:1069–78.
[20] Sipos D, Chandrasekhar K, Arvidsson K, Engström A, Ehrenberg A. Two-dimensional proton-NMR studies on a hybrid peptide between cecropin A and melittin. Eur J Biochem 1991;19:285–91.
[21] Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008;3:163–75.
[22] Park CB, Kim HS, Kim SC. Mechanism of action of the antimicrobial peptide Buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem Biophys Res Commun 2008;244:253–7.
[23] Ulvatne H, Samuelsen Ø, Haukland HH, Krämer M, Vorland LH. Lactoferricin B inhibits bacterial macromolecular synthesis in *Escherichia coli* and *Bacillus subtilis*. FEMS Microbiol Lett 2004;237:377–84.
[24] Cassone M, Otvos Jr L. Synergy among antibacterial peptides and between peptides and small-molecule antibiotics. Expert Rev Anti Infect Ther 2010;8:703–16.
[25] Mani R, Cady SD, Tang M, Waring AJ, Lehrer RI, Hong M. Membrane-dependent oligomeric structure and pore formation of a β-hairpin antimicrobial peptide in lipid bilayers from solid-state NMR. Proc Natl Acad Sci U S A 2006;103:16242–7.
[26] Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, et al. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature 2008;455:804–7.
[27] Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology 2010;156:3216–23.
[28] Williams DF. On the mechanisms of biocompatibility. Biomaterials 2008;29:2941–53.
[29] Bell G, Gouyon PH. Arming the enemy: the evolution of resistance to self-proteins. Microbiology 2003;149:1367–75.
[30] Jin Y, Hammer J, Pate M, Zhang Y, Zhu F, Zmuda E, et al. Antimicrobial activities and structures of two linear cationic peptide families with various amphipathic β-hairpin and α-helical potentials. Antimicrob Agents Chemother 2005;49:4957–64.
[31] Jiang Z, Vasil AI, Gera L, Vasil ML, Hodges RS. Rational design of α-helical antimicrobial peptides to target Gram-negative pathogens, Acinetobacter baumannii and Pseudomonas aeruginosa: utilization of charge, 'specificity determinants', total hydrophobicity, hydrophobe type and location as design parameters to improve the therapeutic ratio. Chem Biol Drug Des 2011;77:225–40.
[32] Dathe M, Wieprecht T. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochim Biophys Acta 1999;1462:71–87.
[33] Giangaspero A, Sandri L, Tossi A. Amphipathic α-helical antimicrobial peptides. Eur J Biochem 2001;268:5589–600.
[34] Andreu D, Ubach J, Boman A, Wåhlin B, Wade D, Merrifield RB, et al. Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. FEBS Lett 1992;296:190–4.
[35] Chicharro C, Granata C, Lozano R, Andreu D, Rivas L. N-terminal fatty acid substitution increases the leishmanicidal activity of CA(1-7)M(2-9), a cecropin-melittin hybrid peptide. Antimicrob Agents Chemother 2001;45:2441–9.
[36] Fox MA, Thwaite JE, Ulaeto DO, Atkins TP, Atkins HS. Design and characterization of novel hybrid antimicrobial peptides based on cecropin A, LL-37 and magainin II. Available from: Peptides. http://dx.doi.org/10.1016/j.peptides.2012.01.013 http://www.sciencedirect.com/science/article/pii/S0196978112000344; 2012.
[37] Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS. Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides. Antimicrob Agents Chemother 2007;51:1398–406.
[38] Kim S, Kim SS, Lee BJ. Correlation between the activities of alpha-helical antimicrobial peptides and hydrophobicities represented as RP HPLC retention times. Peptides 2005;26:2050–6.
[39] Laverty G, McLaughlin M, Shaw C, Gorman SP, Gilmore BF. Antimicrobial activity of short, synthetic cationic lipopeptides. Chem Biol Drug Des 2010;75:563–9.
[40] Sato H, Feix JB. Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic alpha-helical antimicrobial peptides. Biochim Biophys Acta 2006;1758:1245–56.
[41] Hsu CH, Chen C, Jou ML, Lee AY, Lin YC, Yu YP, et al. Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. Nucleic Acids Res 2005;33:4053–64.
[42] Cirioni O, Giacometti A, Kamysz W, Silvestri C, Riva A, Della Vittoria A, et al. In vitro activities of tachyplesin III against *Pseudomonas aeruginosa*. Peptide 2007;28:747–51.
[43] Gobbo M, Benincasa M, Bertoloni G, Biondi B, Dosselli R, Papini E, et al. Substitution of the arginine/leucine residues in apidaecin Ib with peptoid

residues: effect on antimicrobial activity, cellular uptake, and proteolytic degradation. J Med Chem 2009;52:5197–206.
[44] Nguyen LT, Chau JK, Perry NA, de Boer L, Zaat SA, Vogel HJ. Serum stabilities of short tryptophan- and arginine-rich antimicrobial peptide analogs. PLoS One 2010;5:e12684.
[45] Rozek A, Powers JP, Friedrich CL, Hancock RE. Structure-based design of an indolicidin peptide analogue with increased protease stability. Biochemistry 2003;42:14130–8.
[46] Lai YP, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. Cell 2009;30:131–41.
[47] Rosenfeld Y, Shai Y. Lipopolysaccharide (Endotoxin)-host defense antimicrobial peptides interactions: role in bacterial resistance and prevention of sepsis. Biochim Biophys Acta 2006;1758:1513–22.
[48] Zughaier SM, Shafer WM, Stephens DS. Antimicrobial peptides and endotoxin inhibit cytokine and nitric oxide release but amplify respiratory burst response in human and murine macrophages. Cell Microbiol 2005;7:1251–62.
[49] Nagaoka I, Hirota S, Niyonsaba F, Hirata M, Adachi Y, Tamura H, et al. Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-alpha by blocking the binding of LPS to CD14(+) cells. J Immunol 2001;167:3329–38.
[50] Nan YH, Bang JK, Shin SY. Design of novel indolicidin-derived antimicrobial peptides with enhanced cell specificity and potent anti-inflammatory activity. Peptides 2009;30:832–8.
[51] Motzkus D, Schulz-Maronde S, Heitland A, Schulz A, Forssmann WG, Jübner M, et al. The novel β-defensin DEFB123 prevents lipopolysaccharide-mediated effects in vitro and in vivo. FASEB J 2006;20:1701–2.
[52] Buxadé M, Lunazzi G, Minguillón J, Iborra S, Berga-Bolaños R, Del Val M, et al. Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFA T5. J Exp Med 2012;209:379–93.
[53] Niyonsaba F, Ushio H, Nakano N, Nq W, Sayama K, Hashimoto K, et al. Antimicrobial peptides human β-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. J Invest Dermatol 2007;127:594–604.
[54] Brueckmann M, Hoffmann U, Dvortsak E, Lang S, Kaden JJ, Borggrefe M, et al. Drotrecogin alfa (activated) inhibits NF-kappa B activation and MIP-1-alpha release from isolated mononuclear cells of patients with severe sepsis. Inflamm Res 2004;53:528–33.
[55] Weber NC, Blumenthal SB, Hartung T, Vollmar AM, Kiemer AK. ANP inhibits TNF-α-induced endothelial MCP-1 expression-involvement of p38 MAPK and MKP-1. J Leukoc Biol 2003;74:932–41.