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ORIGINAL ARTICLE

Rational design of cationic antimicrobial peptides by the tandem of leucine-rich repeat

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Abstract Antimicrobial peptides represent ancient host defense effector molecules present in organisms across the evolutionary spectrum. Lots of antimicrobial peptides were synthesized based on well-known structural motif widely existed in a variety of lives. Leucine-rich repeats (LRRs) are sequence motifs present in over 60,000 proteins identified from viruses, bacteria, and eukaryotes. To elucidate if LRR motif possesses antimicrobial potency, two peptides containing one or two LRRs were designed. The biological activity and membrane–peptide interactions of the peptides were analyzed. The results showed that the tandem of two LRRs exhibited similar antibacterial activity and significantly weaker hemolytic activity against hRBCs than the well-known membrane active peptide melittin. The peptide with one LRR was defective at antimicrobial and hemolytic activity. The peptide containing two LRRs formed α-helical structure, respectively, in the presence of membrane-mimicking environment. LRR-2 retained strong resistance to cations, heat, and some proteolytic enzymes. The blue shifts of the peptides in two lipid systems correlated positively with their biological activities. Other membrane-peptide experiments further provide the evidence that the peptide with two LRRs kills bacteria via membrane-involving mechanism. The present study increases our new understanding of well-known LRR motif in antimicrobial potency and presents a potential strategy to develop novel antibacterial agents.

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Abbreviations

AMP	Antimicrobial peptide
LRR	Leucine-rich repeat
diSC3(5)	3-3-Dipropylthiadicarbocyanine-iodide
MIC	Minimum inhibitory concentration
MHC	Minimal hemolytic concentration
CD	Circular dichroism
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
NPN	N-phenyl-1-naphthylamine
MH	Mueller–Hinton

Introduction

Alexander Fleming's discovery of penicillin in 1928 saved countless lives in less than nine decades. However, human deaths stemming from infectious diseases have steadily reemerged in parallel with the rise of antibiotic-resistant pathogens, and it is urgent to develop new classes of antibiotics (Hasper et al. 2006). Possibly, natural antimicrobial compounds in self-immune systems have the potential when they are used therapeutically. Antimicrobial peptides (AMPs) could represent such a new class of antibiotics. AMPs are less likely to promote similar resistance problem because a microbe would have to redesign its membrane, changing the composition and/or organization of its lipids, probably a 'costly' solution for most microbial species (Zasloff 2002). Antimicrobial peptides

are ancient host defense effector molecules present in organisms across the evolutionary spectrum (Yeaman and Yount 2003).

AMPs are generally defined as having 10 to about 50 amino acids with 2–9 positively charged lysine or arginine residues and up to 50 % hydrophobic amino acids (Brown and Hancock 2006). AMPs display a large heterogeneity in primary and secondary structures but share common features such as amphipathy and net positive charge (Navon-Venezia et al. 2002; Ahmad et al. 2006). The factors believed to influence the potency and spectrum of activity include peptide size, hydrophobicity, amphipathy, and positive charge (Zelezetsky and Tossi 2006). These features of AMPs are often in accord with the characteristics of common protein structural motif. For example, AMPs have been designed and synthesized based on the leucine zipper sequence (Ahmad et al. 2006, 2011). The well-known and widespread structural motif leucine-rich repeat (LRR) also has the common characteristics of being unusually rich in the hydrophobic amino acid leucine and the ability to form amphipathic structures. LRRs are present in over 60,000 proteins that have been identified in viruses, bacteria, and eukaryotes (Matsushima et al. 2010), which are usually present in tandem (Matsushima et al. 2000). The defining feature of the LRR motif is an 11-residue consensus sequence LxxLxLxxNxL (x being any amino acid) (Matsushima et al. 2010; Bellaa et al. 2008). Typically, each repeat unit has a variety of structural elements (a-helices, β-turns and even short β-strands), and the assembled domain composed of many such repeats has a curved solenoid shape with an interior parallel beta sheet and an exterior array of helices (Bellaa et al. 2008).

In the present study, we developed two antibacterial peptides containing one or two LRRs. Using independent methods, i.e. confocal microscopy and flow cytometry, we found that the tandem of two LRRs kill bacteria via membrane-mediating mechanism similar with well-accepted membrane-damaging peptide melittin. The study of membrane-peptide interactions by mimicking the cell membrane with liposome further provided the evidence. The peptide with two LRRs is indeed effective against bacteria.

Materials and methods

Peptide synthesis

The peptides and FITC-labeled peptides were purchased from GL Biochem Corporation (Shanghai, China) and were synthesized by solid-phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. The peptides were amidated at the C terminus. The purity of the peptides (>95 %) was assessed by reverse-phase high-performance liquid chromatography, and the peptides were further subjected to electrospray mass spectrometry to confirm their molecular weight.

Antimicrobial assays

Minimum inhibitory concentration (MIC) testing was performed by a modified version of the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method as described previously (Steinberg et al. 1997). In brief, bacteria were grown overnight in Mueller–Hinton (MH) broth at 37 °C. Then the cultures were diluted to $\sim 1 \times 10^5$ CFU/ml. The peptides dissolved in 0.01 % (v/v) acetic acid and 0.2 % (w/v) bovine serum albumin (Sigma) were added to each well of 96-well plates at the final concentrations ranging from 0.25 to 128 μM. Each well contained a total volume of 100 μl (50 μl of inoculum and 50 μl of peptide-containing solution). MICs were determined as the lowest concentration of peptide that prevented visible turbidity by visual inspection after incubation at 37 °C for 20–24 h. Independent experiments were carried out three to five times. Uninoculated MH broth was used as a negative control and cultures without added peptides served as the positive control.

Measurement of hemolytic activity

The hemolytic activity of the peptides was measured as the amount of hemoglobin released by the lysis of human erythrocytes (Stark et al. 2002). Fresh human red blood cells (hRBCs) were obtained by centrifuging whole blood at $1{,}000\times g$ for 5 min at 4 °C. The erythrocytes were washed three times with phosphate-buffered saline (PBS) and then resuspended in PBS. The peptides dissolved in PBS (50 μl) were added to the hRBCs solution (50 μl). The suspension was incubated for 1 h at 37 °C. Intact erythrocytes were pelleted by centrifugation at $1{,}000\times g$ for 5 min at 4 °C, and the release of hemoglobin was monitored by measuring the absorbance at 492 nm. Peptide concentrations causing 50 % hemolysis were used as the minimal hemolytic concentration (MHC). Zero hemolysis (blank) and 100 % hemolysis were determined by incubating the cells with PBS and 0.1 % Triton X-100, respectively.

The effects of salts on the antimicrobial activity of the peptides

The stability of the peptides was tested in the MIC assay mentioned above with $\sim 5 \times 10^4$ CFU/ml of *E. coli* ATCC 25922. Each well contains final concentrations of NaCl in the range of 50–150 mM, or $MgCl_2$ or $CaCl_2$ fixed at

1 mM to evaluate the salt stability of the peptides. The solutions with dilution of bacteria at salts ($CaCl_2$, $MgCl_2$, or NaCl) without peptides were conducted as control.

The effects of heat and enzymes on the antimicrobial activity of the peptides

To test thermal stability and susceptibility to enzymes, the peptides were processed in advance. For heat resistance experiment, the peptides were incubated at 100 °C for 30 min and cooled on ice for 10 min. For the sensitivity of the peptides to proteolytic enzymes, a solution of the peptide was incubated at 37 °C for 1 h with 1 mg/ml final concentration of the following enzymes: trypsin, pepsin, caroid, and proteinase K. After the treatment, the procedures are the same as described above.

CD analysis

Circular dichroism (CD) spectra of the peptides were measured at 25 °C using a J-820 spectropolarimeter (Jasco, Tokyo, Japan). The peptides were dissolved in 10 mM sodium phosphate buffer, pH 7.4, 50 % TFE, or 25 mM SDS to a final concentration of 125 μM. The solutions were loaded into a 0.1-cm path length rectangular quartz cell, and the spectra were recorded between 190 and 250 nm at 0.5 nm increments. The average mean residue ellipticities were plotted against the wavelength (in nanometers).

Preparation of liposomes

Small unilamellar vesicles (SUVs) were prepared for fluorescence spectroscopy as described previously (Lee et al. 2006; Yang et al. 2003). Egg yolk L-α-phosphatidylcholine (PC), egg yolk L-α-phosphatidyl-DL-glycerol (PG), egg yolk L-α-phosphatidylethanolamine (PE), cholesterol, and acrylamide were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Briefly, PE/PG (7:3, w/w) or PC/cholesterol (10:1, w/w) lipids were dissolved in chloroform, dried with a stream of nitrogen, and lyophilized overnight. Dried lipid films were resuspended in 10 mM sodium phosphate buffer, pH 7.4, vigorously vortexed, and sonicated in ice-cold water for 20 min using an ultrasonic cleaner until the solutions became clear.

Tryptophan fluorescence

Tryptophan fluorescence experiment was used to assess the interaction of the peptides with SUVs of varying lipid composition. The tryptophan fluorescence spectra were measured using an F-4600 fluorescence spectrophotometer (Hitachi, Japan). The fluorescence emission spectra were measured in 10 mM sodium phosphate buffer (pH 7.4) as well as in the presence of negatively charged PE/PG (7:3, w/w) SUVs or zwitterionic PC/cholesterol (10:1, w/w) SUVs. The peptide/liposome mixture (molar ratio of 1:200) was incubated at 25 °C for 10 min. The fluorescence was excited at 280 nm and emission was scanned from 300 to 400 nm.

Evaluation of outer membrane permeability

The ability of the peptides to disrupt bacterial outer membrane integrity was evaluated using the fluorescent dye N-phenyl-1-naphthylamine (NPN) as previously described (Loh et al. 1984). Briefly, *E. coli* UB1005 cells were suspended in 5 mM sodium HEPES buffer, pH 7.4, containing 5 mM glucose. NPN was added to log-phase bacteria at a final concentration of 10 μM. The stabilized background fluorescence was recorded (excitation λ 350 nm, emission λ 420 nm) at 25 °C. The peptide was added to the quartz cuvette, and the increase in fluorescence was recorded.

Evaluation of inner membrane permeability

The cytoplasmic membrane depolarization activity of the peptides was measured using *E. coli* and the cyanine $diSC_3(5)$ as previously described (Wu et al. 1999). Briefly, the bacteria were centrifuged and washed with buffer (20 mM glucose, 5 mM HEPES, pH 7.3) and resuspended to an OD_{600} of 0.05 in the same buffer. The cell suspension was incubated with 0.4 μM $diSC_3(5)$ until a stable reduction of fluorescence was achieved (around 1 h). Then KCl was added to a final concentration of 0.1 M to equilibrate K^+ levels. The peptides were added to achieve the desired concentrations. Changes in fluorescence were recorded using an F-4600 fluorescence spectrophotometer (Hitachi, Japan) with an excitation wavelength of 622 nm and an emission wavelength of 670 nm at 25 °C.

Confocal laser scanning microscopy

E. coli (ATCC 25922) cells were grown to the mid-logarithmic phase and washed three times with 10 mM PBS. Next, *E. coli* (10^7 CFU/ml) cells were incubated with FITC-labeled peptides at MIC and 2MIC at 37 °C for 30 min. The bacterial cells were washed three times with PBS by centrifugation. A smear was made, and images were captured using a Leica TCS SP2 confocal laser-scanning microscope with a 488-nm band-pass filter for FITC excitation.

Table 1 The sequence of the peptides

Peptide	Amino acid sequence[a]	Molecular weight (Da)	Net charge
LRR-1	LRRLWLRANRL	1,465.83	+5
LRR-2	$(LRRLWLRANRL)_2$	2,914.62	+9
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	2,846.49	+6

[a] The peptides were aminated at the C terminus

Table 2 MICs of the peptides

Strains	MIC (μM)		
	LRR-1	LRR-2	Melittin
Gram-negative bacteria			
E. coli ATCC 25922	128	4	1
P. aeruginosa ATCC 27853	>128	4	2
S. typhimurium C7731	>128	2	2
Gram-positive bacteria			
S. epidermidis ATCC12228	>128	2	0.5
E. faecalis ATCC 29212	>128	2	1
S. aureus ATCC 29213	128	2	1

Flow cytometry

E. coli (ATCC 25922) cells were harvested at log phase and washed thrice with 10 mM PBS. Cells were mixed with the peptides (MIC, 2MIC, and 4MIC) at 37 °C for 30 min. Then the solution was incubated with 25 μg/ml propidium iodide (PI, Sigma) at 4 °C for 30 min. The bacterial cells were centrifugated and resuspended in PBS. Flow cytometry was performed using a FACScan (Becton–Dickinson, San Jose, CA). *E. coli* cells were incubated with PI without peptide treated as negative control.

Results

Peptide design

To elucidate if LRR motif has antimicrobial potency, two peptides containing one LRR or tandems of two LRRs were designed (Table 1). This novel LRR motif has 11 residues with the sequence of LRRLWLRANRL, which has four leucines and four positively charged arginines. Generally, it is widely recognized that the initial electrostatic charge attraction is required for peptide–membrane interactions while the varying depths of penetration into the cell membrane depend on the overall hydrophobicity of the peptides (Yeaman and Yount 2003; Zelezetsky and Tossi 2006).

Antimicrobial and hemolytic activities of the peptides

The minimal inhibitory concentrations of the two peptides are shown in Table 2. LRR-1 exhibited activity against *E. coli* and *S. aureus* with MICs for 128 μM while no antimicrobial activity was observed for other microorganisms at tested concentrations. LRR-2 displayed strong antibacterial activity similar to the well-known peptide melittin. The tandem of two LRRs exhibited antimicrobial activity against Gram-negative and Gram-positive bacteria with MICs in the range of 2 to 4 μM, which was at least 32 times larger than that of monomer peptide.

To further look into the cytotoxic activity of the peptides against mammalian cells, their hemolytic activity was also tested against the highly sensitive human erythrocytes. Figure 1 shows that LRR-1 was devoid of hemolytic activity up to 128 μM (the maximum concentration tested). However, LRR-2 exhibited hemolytic activity in a dose-dependent manner. MHCs of LRR-2 were about 36-fold larger than that of melittin, with 66 and 1.8 μM, respectively.

The resistance to salts, enzymes, and heat of the peptides

LRR-2 is chosen to investigate their resistance to salts, enzymes, and heat. In the environment of 1 mM $CaCl_2$ or $MgCl_2$, a series of NaCl concentrations ranged from 50 to 150 mM, the peptide LRR-2 retained its antimicrobial activity with MICs at 4 μM, which suggests that antibacterial potency was not affected by monovalent or divalent cations. Moreover, LRR-2 exhibited strong thermal stability and antimicrobial activity against *E. coli* remained stable after 30 min of incubation at 100 °C. Trypsin and caroid did not affect the antimicrobial activity, with MICs at 4 μM. However, proteinase K and pepsin retain part of

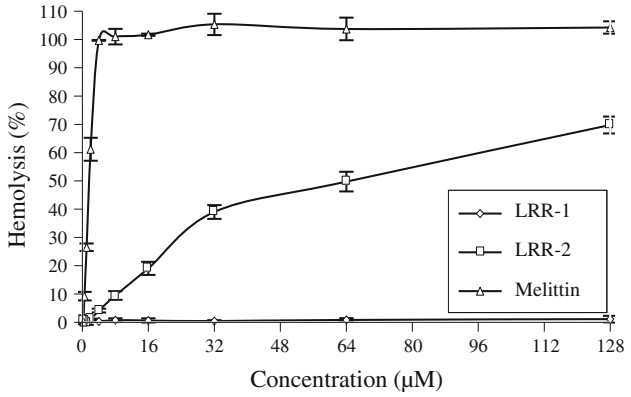

Fig. 1 The hemolysis against hRBCs of the peptides. Peptide concentrations causing 50 % hemolysis for 1 h at 37 °C were used as the minimal hemolytic concentration (MHC)

the antimicrobial activity of LRR-2, with MICs at 32 and 64 μM, respectively.

Secondary structure of the peptides

The conformational preferences of the peptides in different environments were investigated by CD spectroscopy. The hydrophobic and helix-stabilizing agent TFE was used to evaluate the inherent helical propensity of the peptides and SDS micelles with a negatively charged surface was employed to mimic amphiphilic environment of a biological phospholipid bilayer (Park et al. 2001; Wang et al. 1996). As shown in Fig. 2, the CD spectra of the two peptides are characteristic of a random structure (with a minimum near 200 nm) in Tris buffer. In the presence of TFE and SDS, the spectra of LRR-2 displayed showed one positive (~195 nm) and two negative (~208 and 222 nm) bands, which indicate α-helix structure (about 37 % α-helix in TFE and 29 % in SDS). The conformation of LRR-1 in the presence of SDS micelles was the same as that in buffer, with a random coil structure.

Interaction of peptides with model membranes

Tryptophan fluorescence is strongly influenced by the indole side chain and the feasibility of fluorescence spectroscopy for elucidating protein-membrane interactions has been proved (Chapman and Davis 1998). When buried in a hydrophobic environment, Trp fluorescence generally shifts to a shorter (blue shift) maximal wavelength and often exhibits an increase in maximum fluorescence intensity (Ibrahim et al. 2001). Table 3 lists the blue shift values of the peptides in different SUVs. In aqueous solution, the emission maxima for the three peptides were nearly identical (around 350 nm), which is typical for Trp in a hydrophilic environment. Negligible effect was

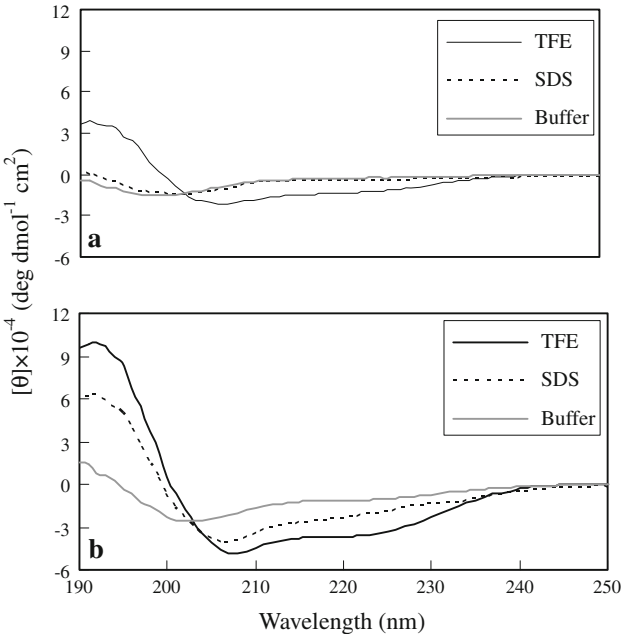

Fig. 2 CD spectra of the peptides. The peptides (**a** for LRR-1 and **b** for LRR-2) with a final concentration of 125 μM were dissolved in 10 mM sodium phosphate buffer, pH 7.4, 50 % TFE, or 25 mM SDS at 25 °C. Mean residue ellipicities were plotted against wavelength. Values from three scans were averaged per sample

observed with addition of both lipid vesicles on the spectrum of the peptide LRR-1. In PE/PG vesicle, the emission maximum decreased by 11 nm for LRR-2 and 18 nm for melittin. Blue shift of LRR-2 was smaller than that of melittin in the presence of PC/cholesterol vesicles, with 9 and 16 nm, respectively. The observed results revealed that the peptide LRR-2 bound preferentially to negatively charged phospholipids over zwitterionic phospholipids, which corresponds well with the biological activity data.

Permeabilization of outer and inner membranes

Peptides were evaluated for their ability to interact with membranes and membrane components in the fluorescence-based NPN uptake and $diSC_3(5)$ release assays. The hydrophobic fluorophor NPN is normally excluded from entering cells due to its inability to penetrate the outer membrane. Upon permeabilization of the outer membrane, NPN is taken up and becomes strongly fluorescent in the hydrophobic environment of cell membranes (Gooderham et al. 2008). Figure 3 shows that the three peptides were able to permeabilize the bacterial outer membrane at tested concentrations from 1 to 8 μM and the increase in fluorescence was similar at the same concentrations. Unexpectedly, LRR-1 was able to permeabilize the outer membrane at the tested concentrations (Fig. 3). But LRR-1

Table 3 Tryptophan fluorescence emission maxima of the peptides in Tris buffer, PE/PG (7:3, w/w), or PC/cholesterol (10:1, w/w) vesicles at a molar lipid/peptide ratio of 200:1 under 25 °C

Peptide	Fluorescence emission maxima (nm)		
	Phosphate buffer	PE/PG	PC/cholesterol
LRR-1	350	349 (1)[a]	350 (0)
LRR-2	350	341 (9)	343 (7)
Melittin	351	335 (16)	337 (14)

[a] Blue shift of emission maximum compared with Tris buffer

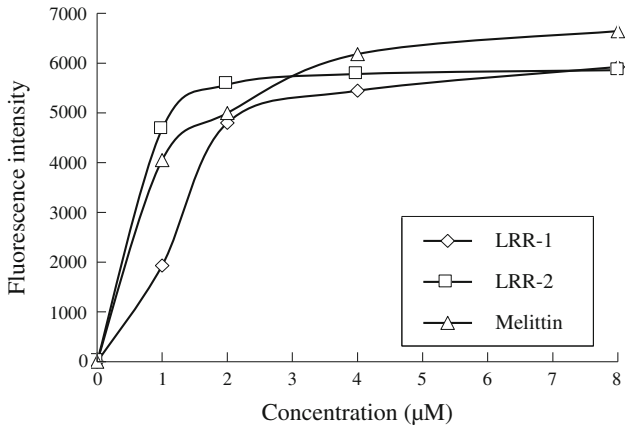

Fig. 3 Uptake of NPN in *E. coli* outer membranes. The ability of peptides (ranged from 1 to 8 μM) to disrupt the outer membrane integrity of bacteria was evaluated with the fluorescent dye N-phenyl-1-naphthylamine (NPN) at a final concentration of 10 μM. Changes in fluorescence were measured using an F-4600 fluorescence spectrophotometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm at 25 °C until the increase was stopped

only displayed poor antimicrobial activity against *E. coli* and *S. aureus* at 128 μM (Table 2).

Peptide-induced changes in the bacterial membrane potential were measured with the potentiometric dye $diSC_3(5)$. The dye $diSC_3(5)$ is known to be distributed between bacterial cells and the surrounding medium, depending on the membrane potential gradient. Once inside the membrane, the dye aggregates and self-quenches. With the addition of a membrane-permeabilizing agent, the dye is released, and the increase in fluorescence can be monitored over time (Pathak and Chauhan 2011). The results showed that the peptides except LRR-1 were able to permeabilize the bacterial cell membrane even at the concentrations lower than their MICs. LRR-1 exhibited defective depolarizing behavior. LRR-2 was more disruptive to the cytoplasmic membrane potential than melittin at the same concentrations. The peptides caused an immediate increase in fluorescence intensity in 50 s, indicating rapid membrane depolarization (Fig. 4).

Together, the results of the outer and inner membrane permeability assays show that LRR-2 had the outer and inner permeabilization activity while the deficiency observed in the antimicrobial activity of LRR-1 against bacteria is primarily a function of its inability to act at the inner membrane.

Membrane–peptide interactions by confocal laser scanning microscopy and flow cytometry

To examine the interaction of the peptide with the bacterial cell membrane in vitro, the localization of FITC-labeled LRR-2 within *E. coli* was visualized under confocal laser-scanning microscopy. As shown in Fig. 5, FITC-derived signals were localized uniformly within the surface of treated cells at the concentrations of MIC and 2MIC, which suggest that FITC-labeled LRR-2 was able to damage the cells by membrane-peptide interactions.

To further detect whether the peptide LRR-2 permeabilizes the cell membrane to permit small dyes into the cytoplasm of bacteria, we tested its effect on bacterial cells incubated with the DNA intercalating dye propidium iodide (PI). If the peptide disrupted the bacterial cell membrane, peptide-induced PI fluorescence would be detected, whereas normal cells showed no PI fluorescence (Park et al. 2006). As shown in Fig. 6, LRR-2 and melittin caused rapid increase in PI fluorescence with 88.6 and 80.4 % cells stained at their respective MIC, respectively. With the increase of peptide concentrations from MIC to 4MIC, the incremental staining ratio was also observed.

Discussion

Here we have derived a novel antibacterial peptide by the tandem of two LRRs. It is reported that the *Listeria monocytogenes* genome includes a large family of proteins harboring leucine-rich repeats known as internalins, which play important functions in the infectious process (Bierne et al. 2007). LRR domains bind particular pathogen-associated molecules, which have been identified as fragments derived from structural components of the pathogens that are functionally important to the life of the organism, such as lipoproteins, peptidoglycans, and lipopolysaccharides

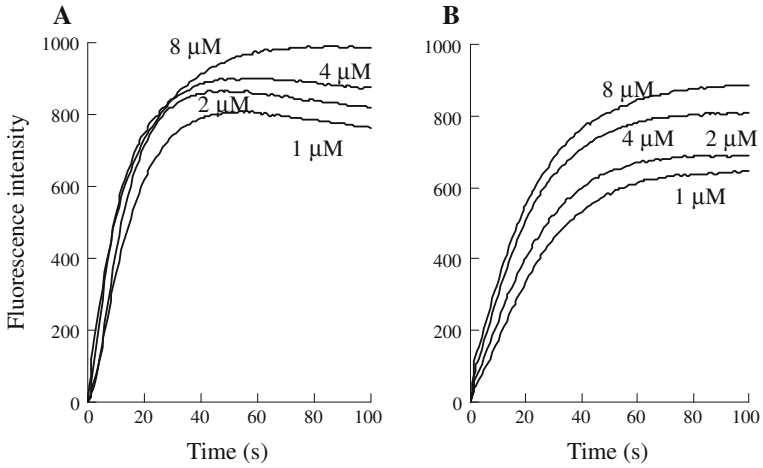

Fig. 4 Cytoplasmic membrane depolarization of *E. coli* by the peptides (**a** for LRR-2 and **b** for melittin). The cytoplasmic membrane permeabilization of the peptides was measured using the cyanine diSC3(5) (0.4 μM). Changes in fluorescence were recorded with an F-4600 fluorescence spectrophotometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm for 100 s at 25 °C

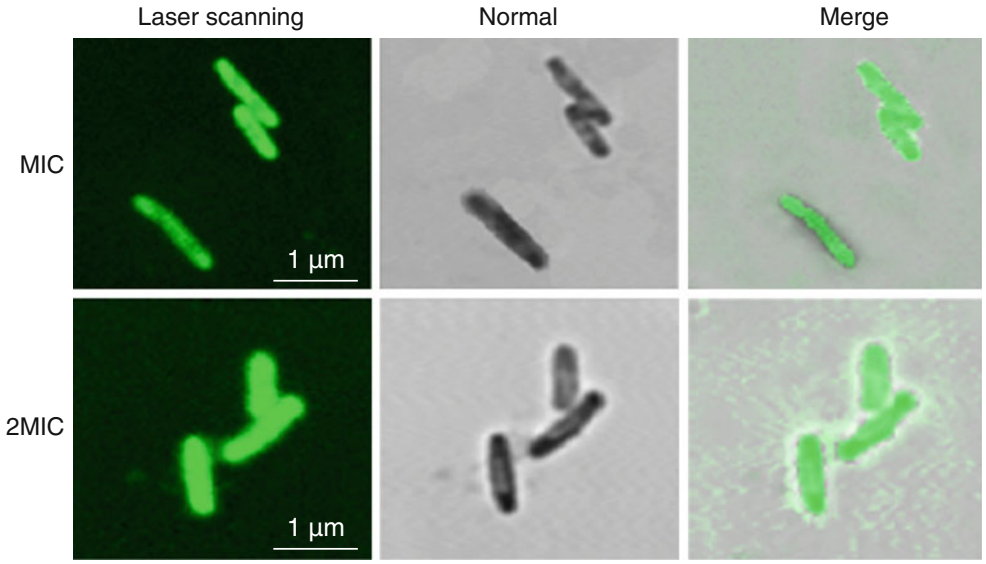

Fig. 5 Confocal laser-scanning microscopy of *E. coli* treated with FITC-labeled peptide LRR-2. The cells were incubated with MIC and 2MIC of FITC-labeled LRR-2 37 °C for 30 min. *Panels* on the *left*, *middle* and *right* represent laser-scanning images, transmitted light-scanning image (normal image), and merged image of *E. coli* treated with FITC-labeled peptides, respectively

(Ryan et al. 2007). LRR proteins encoded in the majority of disease resistance genes are thought to monitor the status of plant proteins that are targeted by pathogen effectors (McHale et al. 2006). Many virulence proteins are detected indirectly by plant LRR proteins while some LRR proteins directly bind pathogen proteins (DeYoung and Innes 2006). These studies warrant further investigation into the LRR motif as a potential antimicrobial agent.

The results showed that the LRR motif has the potential to be the basic element as the antimicrobial agents and the peptide with the tandem of two LRRs exhibited appreciable antibacterial activity and moderate toxicity against hRBCs. LRR-2 displayed strong resistance to salts, heat, and some proteolytic enzymes. Heat stability is potentially a useful characteristic because many procedures such as food or feed processing involve a heating step. An essential requirement for any antimicrobial host defense or therapeutic agent is that it has a selective toxicity for the microbial target relative to the host (Yeaman and Yount 2003). In the present study, the peptide with the tandem of two LRRs exhibited 50 % hemolysis agaiinst hRBCs at 66 μM, which was at least 16 times larger than its MICs. The present study may help in designing and characterizing antimicrobial agent by LRR motif. Leucine zipper motif, also rich in leucine residues, was identified from melittin and plays a crucial role in determining the bactericidal and cytotoxic properties (Asthana et al. 2004; Zhu et al. 2007). Generally speaking, the increase of hydrophobicity or charge is prone to strong toxicity of the peptides (Zelezetsky and Tossi 2006; Yeaman and Yount 2003). Relative hydrophobic moments (mHrel) of LRR-2 and melittin are 0.51 and 0.27, and net charges of LRR-2 and melittin are +9

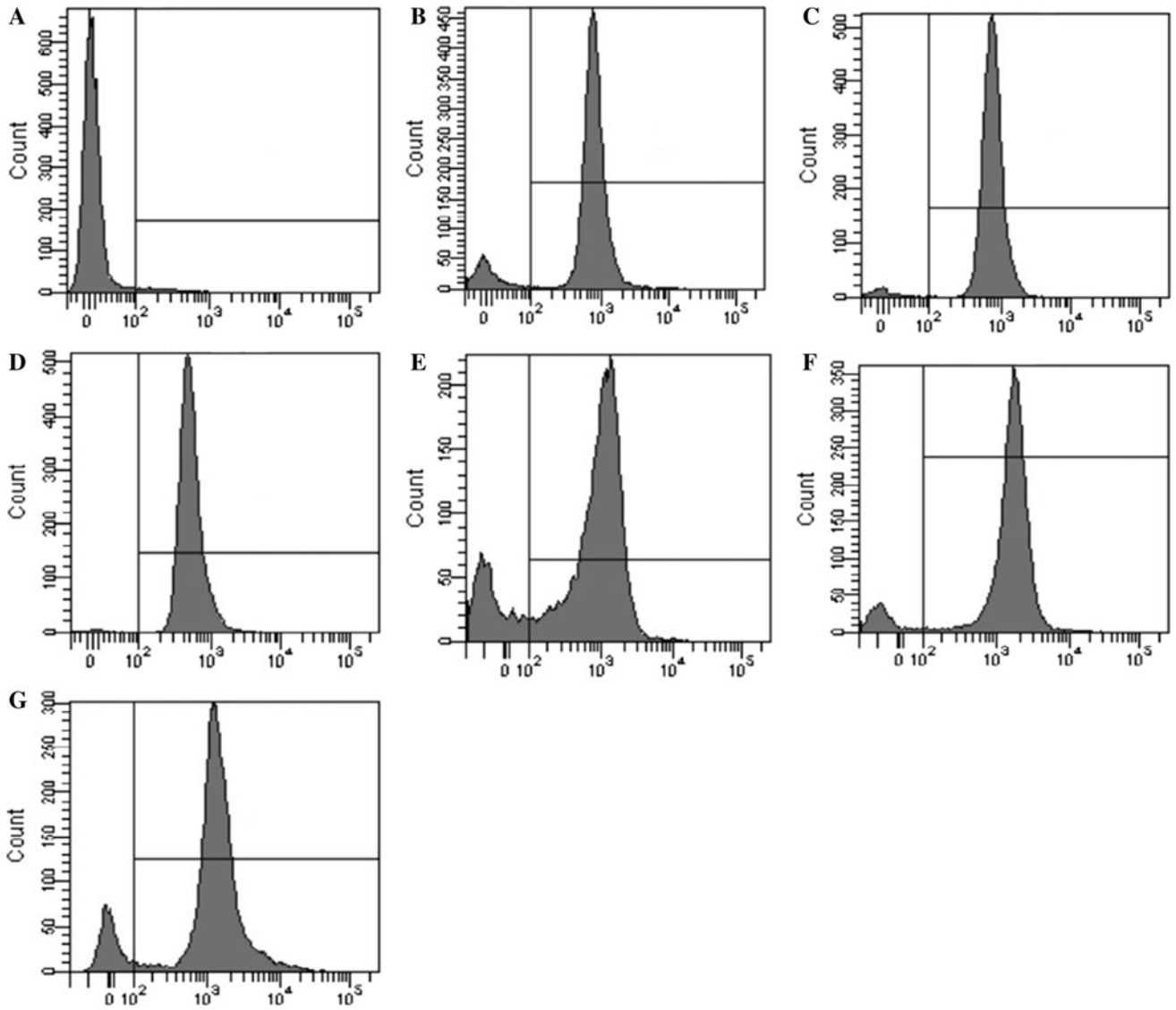

Fig. 6 Flow cytometric analysis. Exponential phase *E. coli* cells were treated with the peptides at 37 °C for 30 min. The cell population showing fluorescence was analyzed by the FACScan. The increments of the log fluorescence signal represent PI uptake by peptides. **a** Without peptide, **b–d** LRR-2 (MIC, 2MIC, 4MIC), **e–g** melittin (MIC, 2MIC, 4MIC)

and +5. Cell selectivity of AMPs may probably be optimized by modulating their assembly (Asthana et al. 2004; Ahmad et al. 2011), which could partly provide explanation about the lower toxicity of LRR-2. Typically, each leucine-rich repeat unit has beta strand-turn-alpha helix structure, and the assembled domain has a horseshoe shape with an interior parallel beta sheet and an exterior array of helices (Matsushima et al. 2010). A single leucine zipper consists of multiple leucine residues at approximately 7-residue intervals, which forms an amphipathic alpha helix with a hydrophobic region running along one side (Landschulz et al. 1988).

The peptide LRR-2 displayed α-helix-rich structures in the presence of membrane-mimicking environments, which corresponds well with its antimicrobial activity. The secondary structure contents of AMPs vary in different lipid environment or at different lipid-to-peptide (L/P) ratios. It would trigger a different amphipathicity, which is the key feature for AMPs to partition in bacterial membranes. At a threshold L/P ratio, peptides orient perpendicular to the membrane. At this point, helices may begin to self-associate, such that their polar residues are no longer exposed to the membrane hydrocarbon chains (Yeaman and Yount 2003). There is a widespread acceptance that the initial mechanism by which antimicrobial peptides target microbes occurs via an electrostatic interaction (Yeaman and Yount 2003). In general, membrane disruption is believed to occur either via a detergent-like carpet

mechanism for short peptides (such as LRR-1), or through insertion into the hydrophobic core of lipids and formation of discrete pores for longer peptides (such as LRR-2) (Zelezetsky and Tossi 2006; Huang 2000). Melittin forms a pore via a barrel-stave mechanism in a zwitterionic membrane (Papo and Shai 2003; Rex and Schwarz 1998) and acts as a detergent in a negatively charged membrane (Papo and Shai 2003; Ladokhin and White 2001).

Tryptophan fluorescence studies indicated that only the peptide with two LRRs penetrated the two lipid systems, whereas LRR-1 was located onto their surface. LRR-1 is rather short and may not be able to span the membrane core as a monomer, whereas LRR-2 could probably fit. It may partly explain the differences observed between the two peptides. Almost negligible blue shift for fluorescence emission of the peptide LRR-1 was consistent with its poor antimicrobial or hemolytic activities. The peptide LLR-2 interacted preferentially with negatively charged phospholipids over zwitterionic phospholipids, which lead to the optimal cell selectivity. These results support an association between antibacterial function of the tandem of LRRs and cell membranes. In addition, LRR-2 showed strong outer and inner membrane permeability activity. Unexpectedly, LRR-1 showed outer membrane permeabilization at low doses, which are several times less than its MICs. But LRR-1 is defective at permeabilize the inner membrane even at the highest tested concentration of 128 μM. Therefore, it is conceivable that the defective antimicrobial activities of LRR-1, as summarized in Table 2, occurred as a consequence of the incapability of inner membrane permeabilization. Confocal laser scanning microscopy showed that FITC-derived signals were localized uniformly within the surface of treated cells. It was reported that FITC-derived signals should be detected exclusively in regions bordering the cell surface if FITC-labeled peptides only associated with the bacterial cell membrane (Ladokhin and White 2001). This suggests that the increased membrane permeability might not be the sole cause of cell death and the peptides (e.g. buforin 2) (Kobayashi et al. 2004) may target intracellular molecules after the damage of cell membranes. Flow cytometry demonstrated that the *E. coli* cell membrane was damaged by peptide LRR-2 and melittin, allowing the free diffusion of PI into the cytoplasm (Park et al. 2006). Collectively, LRR-2 kills bacteria via membrane-mediating mechanism.

In conclusion, our study introduces a novel LRR-containing AMP with a potent antimicrobial activity, which is as effective as well-known peptide melittin. The monomer of LRR displayed poor antimicrobial activity probably as a consequence of defective inner membrane depolarization. CD spectra exhibited that the tandem of two LRRs underwent conformational changes in the presence of membrane-mimetic environment. Membrane-related experiments provide the evidence that the peptide containing LRRs kill bacteria via membrane-mediating mechanism. Overall, the present study not only increases our understanding of the important role of the LRRs in antimicrobial potency, but also presents a potential strategy to develop novel antimicrobial peptides with modulation of their membrane/cell selectivity by altering their structural parameters for future therapeutic purposes.

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