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β-发卡抗菌肽的全新设计及其生物学活性

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摘　要：通过缬氨酸和精氨酸的交替连接形成 β-发卡结构的两条侧链，D-脯氨酸和甘氨酸形成 β-转角单元以及侧链末端的两个半胱氨酸连接形成一个二硫键，来设计得到全新的由 16 残基构成的 β-发卡抗菌肽 VR。对设计得到的抗菌肽 VR 的生物学活性进行了检测，主要测定了新型 β-发卡抗菌肽 VR 的最小杀菌浓度、对红细胞的溶血活性、杀菌动力学和盐敏感性。结果发现，VR 和蜂毒素具有相似的杀菌活性，而溶血活性远低于蜂毒素，这表明 VR 比蜂毒素具有更高的细胞选择性。在 NaCl 的浓度低于 100 mmol/L 时，VR 的杀菌活性没有受到影响；在 NaCl 的浓度为 100 mmol/L 时，VR 具有 50％的杀菌活性。综上可见，VR 具有较优异的生物学活性，拥有成为抗生素替代物的发展潜力。

关键词：抗菌肽，β-发卡，抗菌活性，溶血活性，盐敏感性

Design and biological activity of β-hairpin-like antimicrobial peptide

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Abstract: In the current study, we synthesized a 16-residue-long peptide VR with the aim of inspecting the feasibility to design β-hairpin-like antimicrobial peptide. The peptide was designed by alternating arrangement of arginine and valine and

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linking two stranded antiparallel β-sheet with a short loop segment (^{D}PG) and a disulfide bridge. Antimicrobial and hemolytic activities were investigated. Melittin was chosen as a control peptide. We also tested bactericidal kinetics and salt sensitivity. Results show that VR had similar antibacterial activity compared with melittin. However, VR displayed much less hemolytic activity than melittin. These results suggest that VR had higher cell selectivity than melittin. The antibacterial activity of VR was not inhibited in the presence of 25 and 50 mmol/L NaCl. VR still possessed antibacterial activity in the presence of 100 mmol/L NaCl. Collectively, the de novo peptide VR displayed high antimicrobial activity, low hemolytic activity, and salt resistant, indicating that VR was a promising candidate for novel antimicrobial applications.

Keywords: antimicrobial peptides, β-hairpin, antimicrobial activity, hemolytic activity, salt sensitivity

Introduction

Antimicrobial peptides (AMPs), also called host defence peptides, are an evolutionarily conserved component of the innate immune response. These peptides are potent, broad spectrum antibiotics which demonstrate potential as promising therapeutic agents. AMPs had been demonstrated to kill Gram-negative and Gram-positive bacteria, fungi and enveloped viruses [1]. AMPs are generally less than 50 amino acids and possess cationic and amphipathic features. Although the modes of action by which antimicrobial peptides kill bacteria were not elucidated, they mainly involved in disrupting membranes, interfering with metabolism, and targeting cytoplasmic components [2-3].

The quantitative-structure-activity relationships (QSAR) of AMPs involve the relationship between biological function and secondary structures. Generally, four types of secondary structures are involved: i) α-helical, ii) β-stranded containing two or more disulfide bonds, iii) β-hairpin or loop containing a single disulfide bond and/or cyclization of the peptide chain, and iv) extended [4]. The α-helix and β-sheet are the main secondary structures occurring in known peptide structures. The α-helix has been carefully investigated because there are well-established design principles for creating synthetic peptides [5-8]. However, few studies about β-sheet or β-hairpin-like antimicrobial peptides were performed. Previous study showed that designed short peptides with amphipathic β-hairpin-like structures or two-stranded β-sheet conformations had potent antimicrobial properties and selectivity [9].

In this study, we synthesized a cyclic β-hairpin-like 16-residue-long antimicrobial peptide Ac-CVRVRVR^{D}PGRVRVRVC-NH_2. Arginine (R) is a cationic residue and has a more dispersed positive charge than the single amine of lysine (K), possibly enhancing electrostatic interactions between peptides and the negatively charged bacterial membrane surface [10-11]. Valine (V) is a highly hydrophobic residue, and the V-rich arms in β-sheets are prone to extended strand conformations [12]. A β-turn-nucleating ^{D}PG segment was used to stabilize the observed conformation and it intended to adopt type II′ β-turns, thereby promoting the formation of β-hairpins [13-14]. The antimicrobial and hemolytic properties were measured to evaluate the cell selectivity. We also determined salt sensitivity of the synthetic peptide.

1 Materials and methods

1.1 Peptide synthesis

The peptides VR and melittin were synthesized at GL Biochem Corporation (Shanghai, China) by solid-phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry (Table 1). The peptides were aminated at the C terminus and acetylated at the N terminus. The purity of peptides

was more than 95% by using reverse-phase high-performance liquid chromatography. The peptides were identified by electrospray mass spectrometry.

1.2 Antimicrobial assays

To test the antimicrobial activity, the following strains were used: *Escherichia coli* ATCC 25922, *Salmonella Pullorum* C79-13, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228. Bactericidal activity was determined as described previously [15]. Mid-log-phase bacteria were obtained by growing bacteria in nutrient broth (Bacto; Difco). The bacteria were washed twice with 10 mmol/L sodium phosphate buffer (pH 7.4), and diluted the bacteria solution to 10^6 colony forming units (CFU)/mL in the same buffer. A final volume of 100 μL was used in sterile 96-well plates with different concentrations of the peptides (0.5−128 μmol/L), which was incubated for 2 h at 37 °C. The suitably diluted aliquots were plated on nutrient agar and incubated for 18 h at 37 °C, and colonies were counted. The lowest concentration of peptide at which there was complete killing was taken as the lethal concentration (LC). In the control experiments, cells were incubated with only sodium phosphate buffer. The average of three independent experiments was done in duplicate for LC.

1.3 Kinetics of bacterial killing

The kinetic of bacterial killing was determined against Gram-negative (*E. coli* ATCC 25922) and Gram-positive (*S. aureus* ATCC 29213) bacteria. Mid-log-phase bacteria (10^5 CFU/mL) were separately exposed to the peptide at a final concentration of LC for 0, 2, 5, 10, 30, and 60 min at 37 °C in 10 mmol/L sodium phosphate buffer (pH 7.4). The suitably diluted aliquots were plated on nutrient agar plates. The bacteria were counted after an incubation of 18 h at 37 °C. The values mentioned in the results were the averages of three independent experiments.

1.4 Measurement of hemolytic activity

The hemolytic activity of the peptides was determined by a previously described method [16]. Briefly, fresh human red blood cells (hRBCs) were collected and then centrifuged at 1 000×g for 5 min. The erythrocytes obtained were washed three times with phosphate-buffered saline (PBS), centrifuged for 5 min at 1 000×g, and resuspended in PBS to attain a dilution of about 1% (*V/V*) of the erythrocyte volume initially collected. A volume of 100 μL of hRBCs solution was incubated with 100 μl of serially different peptide dissolved in PBS for 1 h at 37 °C. The peptides were at final concentrations ranging from 0.25 to 256 μmol/L. Intact erythrocytes were pelleted by centrifugation at 1 000×g for 5 min at 4 °C, and supernatant was transferred to a new 96-well microtiter plate. The release of hemoglobin was monitored by measurement of absorbance at 570 nm. As negative and positive controls, hRBCs in PBS and 0.1% Triton X-100 were employed, respectively. MHC was defined as the peptide concentrations causing 5% hemolysis.

1.5 Salt sensitivity

To investigate the effect of salt on antimicrobial activity, salt sensitivity of peptide was measured. NaCl (25 mmol/L, 50 mmol/L, and 100 mmol/L) were added to the incubation buffer to examine the effect of salt on the antibacterial activity of the peptides. The effect of NaCl was examined at the lethal concentration of VR.

1.6 Statistical analysis

Data were analyzed by the ANOVA procedure of the SPSS 16.0 software. Quantitative data was presented as $\bar{x} \pm s$ deviation of the mean. Significant difference was identified at a *P* value of less than 0.05.

2 Results

2.1 Design of peptides

VR was designed by adopting alternate hydrophobic (V) and hydrophilic (R) residues as one of β-hairpin linked by a short two-residue loop segment (^{D}PG) (Table 1; Fig. 1). It was expected that R and V were selected to increase antimicrobial activity and promoted the conformations of extended strand. The central ^{D}PG segment was adopted to be a Type Ⅱ′ β-turn for ideal β-hairpin conformation. The Pro was D-amino acide. VR was stabilized by one disulfide bridge. C-terminus was aminated and N-terminus was protected by using Ac.

2.2 Antimicrobial activity

The antibacterial activities of VR and melittin against Gram-positive and Gram-negative species of bacteria are determined. LCs of VR and melittin were shown in Table 2. The bacterial strains tested were susceptible to VR with LC values in the 1–4 μmol/L range, while melittin was determined at the LC value of 0.5–4 μmol/L. The geometric mean of the LCs of VR and melittin was 1.68 and 1, respectively. These results showed that 16-residue-long VR had similar antimicrobial activity with melittin.

2.3 Kinetics of bacterial killing

The bactericidal activity of the peptide VR occurred more rapid after 5 minutes against *E. coli* ATCC 25922 and 10 minutes against *S. aureus* ATCC 29213 (P<0.01) (Fig. 2). The exposure of VR to a final concentration of 1 μmol/L reduced the colony count by 99% at 30 min.

2.4 Hemolytic activity

We examined the ability of VR in causing the hemolysis of human erythrocytes as a measure of their toxicity to mammalian cells. The hemolytic activities of the VR against human erythrocytes were determined at final peptide concentrations from 0.25 to 256 μmol/L (Table 2). VR displayed 5% hemolytic activity at 256 μmol/L compared with melittin at 0.25 μmol/L.

Table 1 Amino acid sequences and molecular weights of VR and melittin

Peptide	Sequence	Molecular weight (Da)
VR	Ac-CVRVRVR^{D}PGRVRVRVC-NH_2	1 949.30
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2 846.49

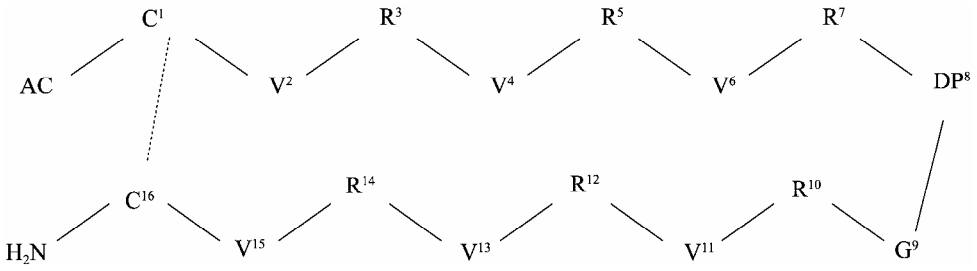

Fig. 1 Sequence and chemical structure of designed cyclic β-hairpin-like cationic peptide (VR) were depicted. Prediction of the secondary structure preferences of the peptide sequences within a protein environment was done with the PHD program [17]. Dashed lines indicate disulfide bridges in VR. Single-letter abbreviations are for the amino acid residues.

Table 2 LC, GM, MHC and therapeutic index of the peptides

Peptide	LC[a] (μmol/L)				GM[b]	MHC[c] (μmol/L)	Therapeutic index[d]
	Gram-negative bacteria		Gram-positive bacteria				
	E. coli	*S. gallinarum*	*S. aureuss*	*S. epidermidis*			
VR	1	4	1	2.0	1.68	256.00	152.38
Melittin	1	2	1	0.5	1.00	0.25	0.25

LC: lethal concentration; GM: geometric mean of LC; MHC: minimal hemolytic concentration. [a] LC is the lowest concentration of the peptide which kills the bacteria completely; [b] The geometric mean of the LCs of the peptides against all four bacteria strains was observed; [c] MHC is the minimal hemolytic concentration that causes 5% hemolysis of human red blood cells (hRBC); [d] Therapeutic index is the ratio of the MHC to the geometric mean of LCs (GM). Larger values indicate greater cell selectivity.

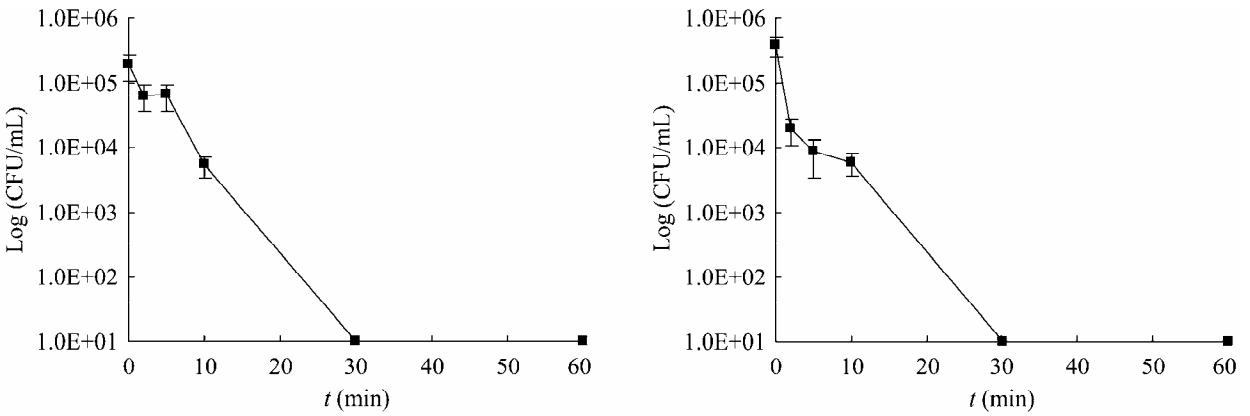

Fig. 2 Time-kill kinetics of the peptide VR against *E. coli* and *S. aureus* was determined with a final concentration of 1 μmol/L.

2.5 Therapeutic index

The ratio of minimal hemolytic concentration (the peptide concentration that causes 5% hemolysis) to GM (the geometric mean of the LCs of bacterial strains observed) was defined as the therapeutic index of VR (Table 2). The therapeutic index of VR was 152.38, which was higher than melittin for 0.25. These results showed that VR displayed a promising therapeutic index.

2.6 Effect of salt on antimicrobial activity

The effects of NaCl on the activities of VR are summarized in Fig. 3. The activity of VR against *E. coli* decreases with increasing concentration of NaCl. When VR was exposed to 25 mmol/L and

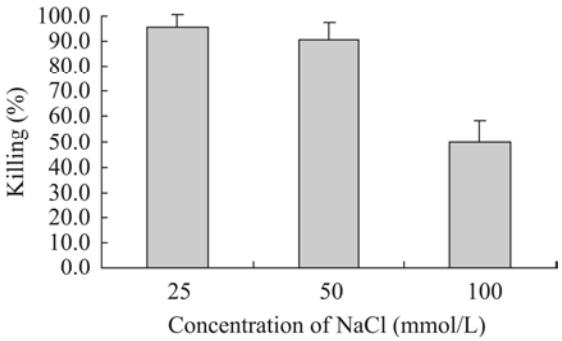

Fig. 3 Mid-log-phase bacteria (10^5 CFU/mL) were incubated with the LC of VR in the presence of 25 mmol/L, 50 mmol/L, and 100 mmol/L. The values reported were the averages of three independent experiments.

50 mmol/L NaCl concentrations, the activity was almost not inhibited. At 100 mmol/L NaCl concentrations, VR still had antibacterial activity, the killing rate was about 50%.

3 Discussion

An important research goal for AMPs is to make them being potential antibiotics substitutes that refer to be effective, low side effects and production costs. Short AMPs are the potential candidates in large-scale chemical peptide production for relatively low synthesis costs. In the present study, the 16-mer peptide VR with seven amino acids in one strand was high potent in killing both Gram-negative and Gram-positive bacteria. Frecer et al[9] designed β-hairpin-like antimicrobial peptides with minimum inhibitory concentrations in the nanomolar range. The geometric mean of the VR's LCs against four bacterial strains was similar with melittin. In addition, rapid bactericidal action is one of the essential features of an effective therapeutic agent. Time-kill kinetics of the VR showed that VR could kill the *E. coli* and *S. aureus* in 30 min, which suggested that VR could be a promising killing agent. In this study, melittin, a 26-residue peptide which is derived from the venom of the European honey bee *Apis mellifera*, was used as a reference to compare with VR. Melittin is an antimicrobial peptide with commonly used as a cell-lysing agent. It is one of the most widely studied membrane-active peptides [18]. VR showed almost no cytotoxicity on human erythrocytes, while melittin exhibited a potent hemolytic activity regarding most tested concentration amounts. Therefore, these results demonstrated that VR had higher cell selectivity between microbial cells and mammalian cells compared to melittin.

AMPs with β-hairpin-like structures or two-stranded β-sheet conformations had potent antimicrobial properties and high cell selectivity [9,19-20]. The ^{D}PG segment was a very strong inducer of β-sheet formation [21-22] and was used to achieve the cyclization of backbone. Furthermore, disulfide cyclization induced the formation of a superimposable β-turn in aqueous solution [23]. Therefore, VR was inclined to conduct a β-hairpin-like conformation. In this study, VR had potent antimicrobial properties and high cell selectivity, which may be closely related to its secondary structure.

Heather and Faisal found that two-stranded β-sheet (''β-hairpin'') became more stable when the length of strands were seven residues, and longer strands did not cause the stability improved [24]. In the study, VR with seven residues in each strand showed a high antibacterial activity. Further work needs to be done to find out the relationship between antibacterial activity and stabilization of two-stranded β-sheet antibacterial activity.

Salt inactivation of antibacterial activity is dependent on the concentration of NaCl. In this study, the antibacterial activity of VR was not inhibited in the presence of 25 and 50 mmol/L NaCl, while antibacterial activity of VR was decreased by 50% at 100 mmol/L. The peptide VR showed a similar result with the carboxy-terminal region of human β-defensins (HBD1–3) with a single disulfide bond [25]. Human β-defensins 3 with three disulfide bridges exhibited activity against Gram-negative and Gram-positive bacteria, which was not inhibited in the presence of physiological concentrations of NaCl [26]. It was conceivable that the net positive charge had a role in modulating antibacterial activity in the presence of NaCl, and the peptides with more net charge displayed stronger salt resistant. In this study, VR with 7 net positive charges had antibacterial activity in the presence of 100 mmol/L NaCl, which suggested that VR was a salt resistant antimicrobial peptide.

4 Conclusion

In summary, the de novo peptide VR contained only 16 amino acids and showed a broad spectrum of activities against Gram-positive as well as Gram-negative bacteria. VR had great antimicrobial activity and low hemolysis, which displayed that VR had high cell selectivity. VR was active in the presence of 100 mmol/L NaCl. Overall, VR could have potential for development as therapeutic agents.

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