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Rational design of α-helical antimicrobial peptide with Val and Arg residues

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Abstract: [**Objective**] The amphipathic α-helical peptide is an important class of antimicrobial peptides. In this study, a 16-residue-long peptide (VGR16) composed of 8 Val residues in the nonpolar face and 5 Arg residues in the polar face was designed based on the helical wheel projection to produce antimicrobial peptide with improved antibacterial activity accompanied by decreased toxicity. [**Methods**] Antimicrobial activity and toxicity against red blood cells and mammalian cells were investigated to evaluate the biological function of the peptide. In addition, bactericidal kinetics was tested. [**Results**] Antimicrobial assays revealed that the peptide VGR16 showed antimicrobial activity and their MICs against gram-negative and gram-positive bacteria ranged from 16 μg/ml to 64 μg/ml. VGR16 also exhibited rapid bactericidal action. It was surprisingly found that the peptide displayed no hemolytic activity even at a concentration of 256 μg/ml. Cell culture assays indicated that the peptide VGR16 had low cytotoxicity against mammalian cells. [**Conclusion**] The results showed that the peptide could be a likely candidate for future antimicrobial applications.

Keywords: antimicrobial peptides, α-helical, residues, hemolysis, cytotoxicity
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The increasing prevalence of multiple-drug resistant pathogens has received widely concerns because of extensive use of antibiotics[1]. Therefore, it is very important to develop novel antimicrobial agents that are capable of overcoming the resistance problem. Antimicrobial peptides (AMPs) are evolutionarily ancient weapons, and their widespread distribution throughout the animal and plant kingdoms suggests that AMPs have served a fundamental role in the successful evolution of multicellular organisms[2]. An important class of AMPs is composed of linear and cationic peptides that form amphipathic α-helices[3]. Many AMPs with cationic and ampihpathic characteristics were designed to study the structure-activity relationships of antimicrobial peptides[4-8].

The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.html) provides us a lot of statistical information including antiviral, antifungal, anticancer and antibacterial peptides. Arg and Val residues occur frequently in hydrophilic and

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hydrophobic amino acids of naturally occurring AMPs. Some AMPs composed of Val and Arg have been designed to synthesize novel peptides with potent antimicrobial activity[5]. In this study, a novel antimicrobial peptide composed of Val, Arg and Gly only (GVVVRVGRVVVRGVRR-amide) were designed with the aim of investigating the feasibility of minimalist approach based on the helical wheel projection (Fig. 1). Similar to the peptides designed by Deslouches et al[5], the percentage of hydrophobic residues was fixed as 50%. Furthermore, residue Gly could be expected to decrease the cytotoxic activity while not affecting antimicrobial potency in this model[8].

1 Materials and methods

1.1 Peptide synthesis

The peptide was designed by GL Biochem Corporation (Shanghai, China) and it was synthesized by solid-phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. The peptide was purified to > 95% purity by reverse-phase high-performance liquid chromatography and its identity was confirmed by electrospray mass spectrometry. The peptide was amidated at the C terminus.

1.2 Antimicrobial assays

Minimum inhibitory concentrations (MICs) of the peptide were measured according to modified version of the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method to determine in vitro antimicrobial activities of peptide as described previously[9]. Briefly, bacterial cells were cultured in Mueller-Hinton (MH) broth overnight and were then diluted to 1×10^5 colony-forming units (CFU)/ml. Serially diluted peptides, dissolved in 0.01% (v/v) acetic acid and 0.2% (w/v) bovine serum albumin (Sigma), were added to each well of the 96-well plates. Then cell suspensions were added to the plate. Plates were incubated at 37℃ overnight, and MICs were determined as the lowest concentrations of the peptide that prevented visible turbidity. As positive control and negative control, cultures without the peptide and uninoculated MH broth were employed.

1.3 Measurement of hemolytic activity

The hemolytic activity of the peptide was determined by a previously described method[10]. Briefly, fresh chicken and human red blood cells (RBCs) were collected and then centrifuged at 1000 × g for 5 min. The erythrocytes obtained were washed three times with phosphate-buffered saline (PBS) and then resuspended in PBS. 50 μl of solutions was incubated with 50μl of serially diluted peptide dissolved in PBS for 1h at 37℃. Intact erythrocytes were centrifugated at 1000 × g for 5 min at 4℃, and release of hemoglobin was monitored by measurement of absorbance at 570 nm. As negative and positive controls, RBCs in PBS and 0.1% Triton X-100 were employed, respectively. MHCs are defined as the peptide concentrations causing 5% hemolysis.

1.4 Cytotoxicity

Cytotoxicity was measured by a colorimetric assay that makes use of a tetrazolium salt, 2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8). Vero cells were kindly provided by College of Veterinary Medicine, Northeast Agricultural University. Vero cells were propagated on 96-well microplates at a density of 2×10^4 cells/well in 100 μl DMEM containing 10% fetal bovine serum. After incubation to over 80% confluence under a fully humidified atmosphere of 95% room air and 5% CO_2 at 37℃, the medium was aspirated and serially diluted peptides were added to cell cultures. Wells containing cells without the peptide served as controls. All groups were further incubated for 24h at the same conditions as before. The WST-8 reagent (10 μl, Dojindo, Japan) was added to each well of a 96 well microplate containing 100μl of cells in the culture medium, and the plate was incubated for 4 h at 37℃. Absorbance was measured at 450 nm using a microplate reader.

1.5 Determination of antimicrobial kinetics

Determination of killing kinetics for the peptide

was conducted as described previously[11]. Briefly, *E. coli* was grown to log phase and diluted to 1×10^5 CFU/ml in LB. Aliquots of bacteria were separately exposed to the peptide at a final concentration of 32 μg/ml for 0, 2, 5, 10, 30, 60, and 90 min at 37℃ under aerobic conditions. A 10μl aliquot was removed at every time point and the surviving CFU were rescued by dilution (1:50) into LB and then spread on LB agar plates for quantitation. Average values of triplicates were expressed as the surviving CFU/ml plotted against time (min).

1.6 Statistical analysis

Vero cell survival and colony count in bactericidal kinetics are presented as mean ± standard deviation of the mean. Statistical comparisons between groups were performed by analysis of variance. Significance was accepted at a P value of less than 0.05.

2 Results

2.1 Design of peptides

Some AMPs, mainly composed of Leu/Lys or Val/Arg[5, 12-14], were successfully designed to produce cationic α-helix AMPs with potential antimicrobial activity. Following those results, it is reasonable to design the antimicrobial peptide VGR16 segregated 5 Arg residues in the polar face or 8 Val residues in the opposite face (Fig. 1). A 16-residue motif was selected considering following reasons: (1) the frequency of net charge (+4 – +6) and hydrophobic residue compositions (50% – 60%) in naturally occurring α-helical AMPs[8]; (2) the length of 16 amino acids was near the estimated number of residues required to form membrane-spanning pores or span a phospholipid bilayer[15].

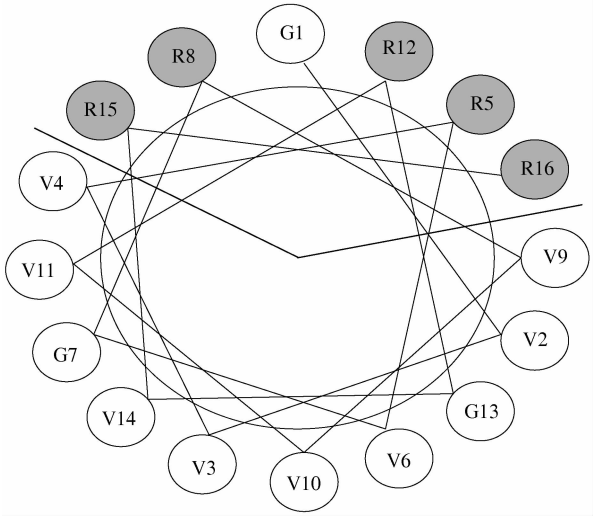

Fig. 1 The sequence template used in this study. Residues are numbered consecutively from the N terminus to the C terminus, with hydrophilic residue (Arg) shaded. According to the helical wheel projection, we designed a new peptide with 8 Val residues in the hydrophobic face and 5 Arg residuesin the hydrophilic face. Residue Gly could be expected to decrease the cytotoxic activity while not affecting antimicrobial potency in this model. Observed molecular weight is 1762.2 Da and net charge is +6.

2.2 Antimicrobial assays

The antimicrobial activity of the peptide VGR16 is shown in Table 1. The peptide displayed different antimicrobial activities against gram-negative and gram-positive bacteria. MICs of the peptide ranged from 16 μg/ml to 64 μg/ml.

Table 1 MICs and MHC of the peptide in this study

Peptide	c(MIC)/(μg/ml)				c(MHCs[a])/(μg/ml)	
	E. coli	*S. typhimurium*	*S. aureus*	*S. epidermidis*	hRBCs	cRBCs
VGR16	32	16	64	16	>256	>256

[a] MHCs are the minimal hemolytic concentrations that caused 5% hemolysis of human red blood cells (hRBCs) or chicken red blood cells (cRBCs).

2.3 Hemolytic activity

The hemolytic activity of the peptide against human and chicken erythrocytes is summarized in Table 1. The peptides VGR16 with 8 Val and 5 Arg residues showed no hemolytic activity against hRBCs and cRBCs even at a concentration of 256 ug/ml, which exceeded the MICs listed in Table 1 by over 4 times.

2.4 Cytotoxicity

The cytotoxic activity of the peptide VGR16 on vero cells at different concentrations is illustrated in Fig. 2. The peptide VGR16 exhibited no significant cytotoxicity at 32 μg/ml. Treatment of vero cells 24 h with 256 μg/ml of VGR16 did not substantially affect

cell viability (about 90%), which exceeds approximately 4-8 times of its MICs.

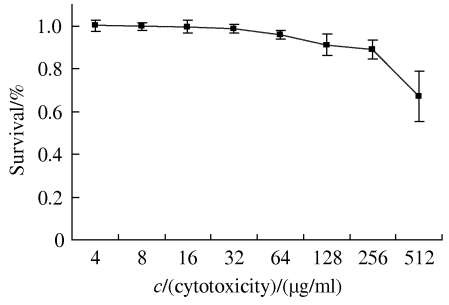

Fig. 2 Cytotoxicity of the peptide VGR16 against vero cells.

2.5 Bactericidal kinetics

Rapid bactericidal action is one of the essential features of an effective therapeutic agent. The bactericidal activity of the peptide VGR16 occurred more rapid after 5 minutes ($P < 0.01$) (Fig. 3). The exposure of VGR16 to a final concentration of 32 μg/ml reduced the colony count by 99% at 60 min.

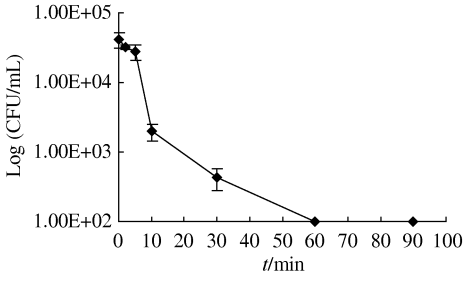

Fig. 3 Time-kill kinetics of the peptide VGR16 against *E. coli* with a final concentration of 32 μg/ml.

3 Discussion

In this study, we successfully engineered a 16-residue-long antimicrobial peptide composed of only Gly, Val, and Arg residues. Firstly we should assume that this linear de novo peptide may form a α-helix. VGR16 inhibited the growth of gram-negative and gram-positive bacteria ranged from 16 μg/ml to 64 μg/ml. In particular, it was gratifying to find that VGR16 had no hemolytic activity even at a final concentration of 256μg/ml, suggesting that multi-Val/Arg residues have huge potential in the design of AMPs with low hemolytic activity. Therefore, further work needs to be done to find out the analogs of VGR16 with enhanced antimicrobial activity.

Deslouches et al designed multimers of a 12-residue lytic base unit (LBU) peptide composed only of Arg and Val residues aligned to form idealized amphipathic helices. Positively charged residues increased from 6 to 24 with the increase of chain length[5], but only 5 Arg residues were used in this study. The difference of positively charged residues may lead to stronger antimicrobial activity of the LBU series. In addition, 3 Gly residues were incorporated into VGR16, which may lead to a decrease of the peptide VGR16[8].

Actually, the rational design in this study corresponds well with the mechanisms of action of antimicrobial peptides[16-17]. Here, we designed 5 negatively charged Arg residues in the polar side of cationic antimicrobial peptides because it is the first step for the positively charged domain of AMPs to interact with the negatively charged components of target membrane by the physical attraction[18-19]. And then we assembled 8 Val residues in the nonpolar face of the peptide considering the intercalation of the hydrophobic domain into the membrane. The de novo peptide showed antibacterial activity but no hemolysis and cytotoxicity may be explicable as follows. Firstly, the differences in the compositions of eukaryotic (higher proportion of zwitterionic phospholipids and uncharged cholesterol) and prokaryotic cell membranes (anionic phospholipids) lead to the failure of interactions of negatively charged Arg residues with eukaryotic membranes[18]. Secondly, 3 Gly residues were designed because the cytotoxicity of AMPs on mammalian cells could be affected by introducing residues capable of increasing the flexibility of the helix (e.g. Gly) at key positions, such as positions 1 or/and 7[8].

In conclusion, amphipathic α-helix AMPs can be designed based on the sequence template. This de novo peptide VGR16 contains only 16 amino acids and showed a broad spectrum of activity including gram-positive as well as gram-negative bacteria. In addition, VGR16 has no hemolysis and low cytotoxicity even at concentration of 256 mg/ml in vitro. Although ongoing

in vivo and in vitro studies are needed to facilitate the design of analogues with other residues substitutions, the results indicated that the peptide VGR16 may be used as a valuable candidate for the development of antibacterial drugs.

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利用精氨酸和缬氨酸设计新型 α-螺旋抗菌肽

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摘要:【目的】抗菌肽是生命体的自身免疫系统的重要组成部分。其中两性的 α-螺旋抗菌肽在抗菌肽家族中又占有重要的地位,发挥着重要的作用。为了得到具有更高抗菌活性同时具有很低细胞毒性的抗菌肽,根据 α-螺旋二级结构衍生出来的螺旋轮模型,设计了一条在疏水一侧含有 8 个缬氨酸和亲水一侧含有 5 个精氨酸的新型 16 残基抗菌肽。【方法】测定了设计得到的新型抗菌肽的最小抑菌浓度、对于红细胞和哺乳动物肾细胞的细胞毒性以及杀菌动力学。【结果】抗菌活性检测表明,新型抗菌肽 VGR16 显示了强并快速的杀菌作用,其最小抑菌浓度在 16 – 64 μg/mL 范围。溶血试验发现抗菌肽 VGR16 在检测的最大浓度 256 μg/mL 处也未见溶血作用。细胞培养试验表明,抗菌肽 VGR16 对非洲猴肾细胞仅在很高浓度时具有很小的细胞毒性。【结论】综上可见,抗菌肽 VGR16 是具有很大抗菌潜力的抗生素替代物。

关键词: 抗菌肽, α-螺旋, 残基, 溶血, 细胞毒性

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