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Potential antibacterial activity of designed *Crocodylus siamensis* hemoglobin-based peptides

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Abstract

In a previous study, we reported an antibacterial peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate, named QL17 (QAIIHNEKVQAHGKKVL); however this peptide has a narrow spectrum of activity. To improve the antimicrobial activity of the peptide, it was used as the template to design novel effective peptides. The helical wheel diagram was used to monitor and evaluate hydrophobicity and hydrophilicity after the positional change and substitution of certain amino acids. Lysine (K) and arginine (R) were appropriately selected to extend the hydrophilicity, whereas hydrophobic residues such as leucine (L), isoleucine (I) or tryptophan (W) were used to increase the hydrophobicity. As appropriate, two novel peptides were synthesized and named as IL-K (IKHWKKVWKHWKKKL) and IL-R (IRHWRRVWRHWRRRL), which had the same hydrophobicity and net charge at 40% and +7, respectively. Evaluation of the antimicrobial activity by broth microdilution assay revealed that IL-K had a slightly higher inhibition activity than IL-R at concentrations of 12.5, 25, 50 and 100 µg/ml. Both peptides had approximately 2-fold percentage inhibition higher than penicillin and as the QL17 parental peptide against *Klebsiella pneumoniae* and *Staphylococcus aureus*. Our findings suggest that the novel designed peptides are promising to be new antibacterial agents for further development and for use as antibiotics.

Introduction

Over the last decade, drug resistance to bacteria has caused serious problems in bacterial infectious disease therapeutics. Thereby, this has become an issue of interest to the World Health Organization (WHO) to find solutions to the problem of drugs being powerless or having limited use. Drug resistance makes the treatment of patients difficult, requiring increased healthcare costs and even unfortunately making it impossible to eradicate certain pathogen bacteria. Accordingly, it is urgently necessary to improve or develop new medical drugs to demolish those resistant bacteria. To overcome this obstacle, antimicrobial peptides (AMPs) are alternative routes to manage resistant bacteria. They are crucial polypeptides in host defense, and stand as a significant weapon in the innate immune system to the potent killing of a broad range of Gram-negative and Gram-positive bacteria, viruses and parasites at low concentrations[1,2,3]. Unlike traditional antibiotic drugs interrupting bacterial growth by various specific intracellular targets, including inhibiting synthesis of DNA, RNA, protein, or cell wall and enzyme inhibitors, on the contrary most AMPs target membranolytic mechanisms

via barrel-stave, carpet and toroidal-pore models to directly disrupt bacterial membrane permeability[4]. In addition to other bacterial killing mechanisms, some AMPs can diffuse inside the cell and eventually inhibit particular intracellular processes[3,5,6]. AMPs generally consist of a short length of 10-50 amino acids along with positive net charges such as +2 to +9. These characteristics help them to react with phospholipid groups of negatively charged bacterial membrane components via electrostatic forces[2]. Moreover, important AMP features enhancing bacterial attack are amphipathicity, in which they should comprise segments of hydrophilic and hydrophobic parts. Such features contribute to AMPs accelerated approach to the bacterial cell membrane and then insert themselves through the cell wall to adhere to phospholipids in the lipid bilayer. When several AMPs cross the bacterial membrane and associate with each other, they can subsequently create membrane leaking, leading to both intrinsic and extrinsic metabolites freely diffusing and bacteria death[4,6].

Our previous studies have reported that a *Crocodylus siamensis* hemoglobin hydrolysate-derived antimicrobial peptide, QL17, displayed MIC_{50} values killing Gram-negative strains *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and Gram-positive strain *Staphylococcus aureus* at concentrations ranging from 10 to 20 mg/ml (w/v). The QL17 peptide containing 17 amino acid residues has 41% hydrophobicity and +2 net charges. The peptide killed bacterial cells through iron dysregulation and an oxidative stress mechanism[7]. However, this peptide has relatively low antibacterial activity.

The improvement of natural peptides to increase the bactericidal rate has been presented by various methods. The first method is *de novo* design that chooses patterns or frequencies of amino acids to create new AMPs without a previously known sequence. But it is restricted to establishing an AMP similar database[8]. The second method is physicochemical, based on structural and chemical properties of amino acid composition[8]. The last method is a template-based design performing by substitution or deletion, particularly in amino acids. It can create novel AMPs with enhanced antimicrobial effect and cell selectivity[2]. Actually, this method needs some information about the physicochemical properties to develop novel AMPs[8]. In the present study, physicochemical and template-based methods were selected to design novel peptides to improve antimicrobial activity.

In this study, QL17 (QAIIHNEKVQAHGKKVL) was used as a peptide template for designing novel peptides with improvement of antimicrobial activity. The QL17 template was substituted with positively charged amino acid residue on a polar segment. The antimicrobial efficiency against Gram-negative and Gram-positive bacteria of peptides was subsequently measured.

Methodology

Peptides design

Antibacterial peptide QAIIHNEKVQAHGKKVL (QL17) from *C. siamensis* hemoglobin hydrolysate with 41% hydrophobicity and +2 net charges was selected as a template for a novel peptide design. The cationicity of a peptide, especially lysine (K) or arginine (R), was used to increase polarity, whereas the hydrophobic residues such as leucine (L), isoleucine (I) or tryptophan (W) were selected to stabilize the peptide structure. The charges of new peptides were designed at around +7 with 40-46% hydrophobicity. The helical wheel projections of QL17 and its analogues were constructed online using the NetWheels: Peptides Helical Wheel and Net projections maker: http://lbqp.unb.br/NetWheels/.

Peptide synthesis

The parent peptide and designed peptides were synthesized using Fmoc solid phase methodology (GL Biochem, Shanghai, China). Peptides were purified by reversed phase high-

performance liquid chromatography. The purity of peptides was greater than or equal to 95%. Then, resulting peptides had their molecular masses confirmed by electrospray ionization-mass spectrometry.

Antimicrobial activity assay

The bacteria used were *Klebsiella pneumoniae* ATCC 27736 and *Staphylococcus aureus* ATCC 25923. Antimicrobial activity of all peptides was determined using the broth microdilution assay. The method was performed according to a previous study[2]. Briefly, bacterial cells were grown to mid-log phase in nutrient broth at 37 °C, and the bacteria were diluted to 10^6 CFU/ml. 50 µl of bacterial suspension was added to each well, together with 50 µl of two-fold serially diluted peptide in a 96-well plate. After incubation at 37 °C for 18-22 h, the value of OD at 600 nm in each well was observed for bacterial growth with a microplate reader (Biochrom, Cambridge, UK). The percentage of inhibition was calculated according to the following formula: % inhibition = $[(OD_{600\ nm,\ control} - OD_{600\ nm,\ sample}) / (OD_{600\ nm,\ control} - OD_{600\ nm,\ blank})] \times 100$. All % inhibition tests were performed in triplicate.

Statistical analysis

Statistical values of all experimental results were calculated using ANOVA, followed by Duncan's test using statistix 8.0. These data are presented as mean ± SD. The *p* value of < 0.05 was considered as being significant.

Results and Discussion

Peptide design and characterization

In this study, the antimicrobial peptide QL17 was used as a template. Novel peptides, namely IL-K and IL-R, were designed by substituting Lys (K) or Arg (R) residues in hydrophilic segments, and Trp (W) residues in hydrophobic segments of parental peptide. However, the replacement of some amino acid residues (Asn, Gln and Gly) in the parent peptide is less essential for membrane-penetrated activity of the peptide[9,10]. Hence, we replaced negatively charged parts and some amino acids with selected amino acids Lys or Arg to increase the hydrophilic area, while Trp was chosen to increase the hydrophobic area. The implementation of our study was to design peptides with preserved facial amphipathic helical parts with an increase of the cationic charge and with a large percentage (≥ 30%) of hydrophobic amino acids[2]. The hydrophilic residues of Lys and Arg were selected to study toxicity, because previous reports have demonstrated that Arg shows toxicity over Lys even when both of them show the same polarity[2]. On the other hand, the selection of Trp was made because the aromatic side chain of Trp can form hydrogen bonds with the phospholipid bilayer of a bacterial cell membrane interface, whereby the peptide is able to permeate deeply and lyse the cell membrane[9,4].

Table 1 shows the sequences and physicochemical properties of the parent peptide and novel peptides. The molecular weights of QL17, IL-K and IL-R were 1913.25, 2073.61 and 2269.70 Da, respectively. Furthermore, the net positive charges on IL-K and IL-R were +7, while, the charge of QL17 was +2. Cationicity is the one crucial features of the antimicrobial efficacy of peptides. Besides, it is involved in interaction formation between cationic peptides and negative charges on the bacterial cell wall, which contain phosphate-head groups of the lipid membrane[11]. On the other hand, the mammalian cell membrane had zwitterionic phospholipids such as phosphatidylcholine, phosphatidylserine and cholesterol that interrupt the attachment of peptides into the membrane[10]. Another feature of AMPs is their hydrophobicity property. Both parent peptide (QL17) and novel peptides (IL-K and IL-R) have a high value of hydrophobicity at 41%, 40% and 40%, respectively. The hydrophobicity of peptides help them to penetrate into the cell membrane, leading to cell membrane pore formation and the occurrence of cell death[9]. However, bulky hydrophobic residues resulted in

strong affinity for mammalian cell membranes and lower cell selectivity[4]. Hence, designed peptides with altered hydrophobic amino acid residues must be considered. The helical wheel projections present the amino acid sequences and properties (Figure 1).

Table 1. Sequences and physicochemical properties of the parent peptide and novel peptides.

Peptide	Sequence	MW (Da)[a]	% Hp[b]	Net charge
QL17	QAIIHNEKVQAHGKKVL	1913.25	41.18%	+2
IL-K	IKHWKKVWKHWKKKL	2073.61	40%	+7
IL-R	IRHWRRVWRHWRRRL	2269.70	40%	+7

[a]Molecular weight (MW) was calculated using an online tool at website https://web.expasy.org/compute_pi/

[b]Percentage of hydrophobicity (% Hp) was calculated using an online tool at website https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php

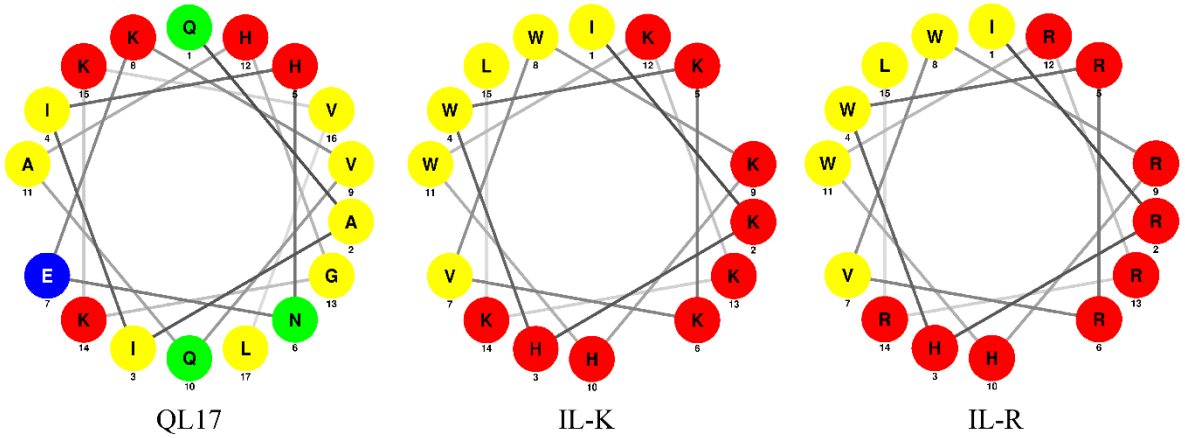

Figure 1. Helical wheel diagrams of the QL17 peptide and its derivative peptides. By default the output presents the polar/basic residues in red, polar/acid residues in blue, polar/uncharged residues in green, and nonpolar residues in yellow.

Antimicrobial activity

The antimicrobial activities of peptides and a conventional antibiotic, penicillin, were evaluated against Gram-negative (*K. pneumoniae*) and Gram-positive (*S. aureus*) bacteria. IL-K and IL-R exhibited higher antimicrobial activity against both Gram-negative and Gram-positive bacteria than penicillin and QL17 at concentrations ranging between 12.5-100 µg/ml (Figure 2). Noticeably, IL-K and IL-R displayed higher antimicrobial activity against *S. aureus* than *K. pneumoniae* at the same concentrations (ranging from 12.5-100 µg/ml). The A Gram-negative bacterium has an outer membrane to protect and reduce the damage induced by a peptide. In contrast, Gram-positive bacteria lack the outer membrane, thus peptides can directly damage cell walls and activate the loss of membrane permeability[4]. The treatment of IL-K on *K. pneumoniae* and *S. aureus* in concentrations of 100 µg/ml displayed a 3-fold and 1-fold percentage inhibition, respectively, compared to parental QL17 and penicillin. Likewise, IL-R showed a 2-fold and 1-fold inhibition against *K. pneumonia*, compared with parental QL17 and penicillin, respectively. In addition, IL-R exhibited increased killing efficiency on *S. aureus* by 3-fold and 2-fold compared with QL17 and penicillin, respectively. The results indicate that IL-K showed significantly better antimicrobial property than IL-R. Decrease in the size of the amino acid side chain may lead to the deep insertion of the peptide into the membrane. The results demonstrate that the positive charge of the peptide plays a major role in antimicrobial efficiency. These investigations are supported by the premise that the addition of positively

charged amino acid residues is efficacious for the initial electrostatic interactions between peptides and negatively charged lipid heads[10,1].

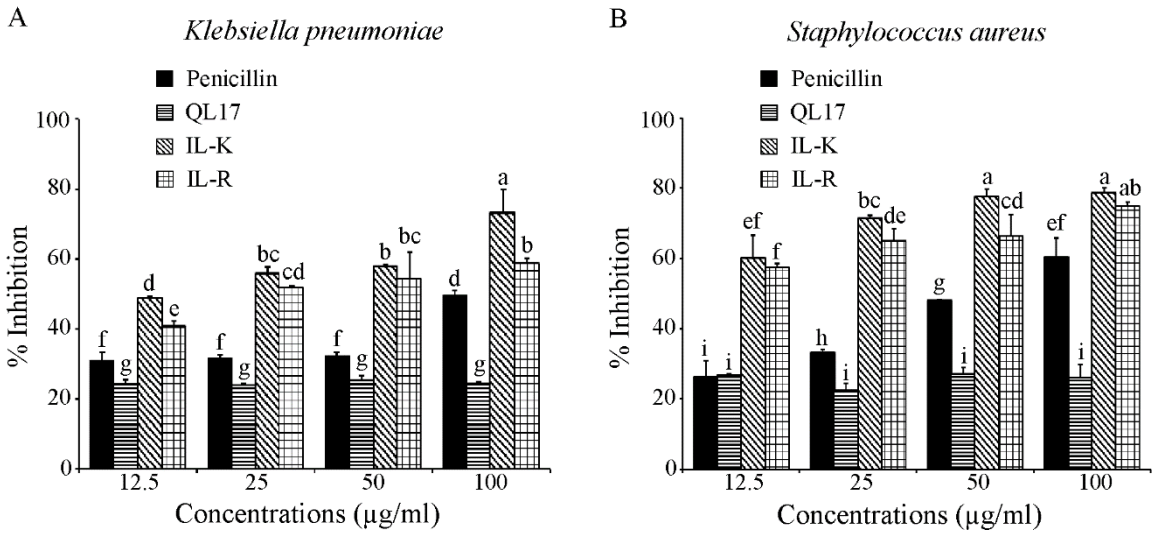

Figure 2. The inhibition percentage of *K. pneumoniae* (A) and *S. aureus* (B) treated with different concentrations of the parent peptide and novel peptides. The letters at the top of each bar indicate significant difference between treatments (p<0.05).

Conclusion

Two novel template-based designed peptides, namely IL-K and IL-R, which were designed by increasing the positive net charges, exhibited an increase in antibacterial property against Gram-negative (*K. pneumoniae* ATCC 27736) and Gram-positive (*S. aureus* ATCC 25923) bacteria when compared to that of a template peptide, QL17. Substitution with Lys residue in the hydrophilic segment of the parent peptide could obtain higher antimicrobial activity than the substitution of Arg. From the results, it is suggested that the size of the peptide affects the bacteria killing mechanism. In addition, a smaller peptide might easily access and insert itself into the membrane, resulting in bacterial cell damage. Hence, the novel designed peptides could be used as an alternative choice for therapeutic agents in the future.

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