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RESEARCH ARTICLE

Effects of lysine-to-arginine substitution on antimicrobial activity of cationic stapled heptapeptides

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Abstract We previously reported a series of amphipathic helices of stapled heptapeptides as membrane-lytic antimicrobial peptides. These peptides possess three lysine residues as the sole cationic amino acid residues in their hydrophilic face of the helix. Lysine-to-arginine substitution is often shown to increase antimicrobial activity of many natural AMPs due to the more favorable interactions of guanidinium moiety of arginine with membranes. In an effort to further improve the pharmacological properties of our novel AMP series, we here examined the impact of lysine-to-arginine substitution on their structures and antimicrobial and hemolytic activities. Our results indicate that the lysine-to-arginine substitution does not always guarantee enhancement in the antimicrobial potency of AMPs. Instead, we observed varied potency and selectivity depending on the number of substitutions and the positions substituted. Our results imply that, in the given helical scaffold stabilized by a hydrocarbon staple, antimicrobial potency and selectivity are influenced by a complex effect of various structural and chemical changes accompanied by lysine-to-arginine substitution rather than solely by the type of cationic residue. These data show potential for use in our scaffold-assisted development of short, selective, and metabolically stable AMPs.

Keywords Antimicrobial peptides · α-Helix · Stapled peptides · Amphipathic peptides · Proteolytic resistance

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Introduction

Amphipathic helical antimicrobial peptides (AMPs) are a well-known class of the membrane-lytic antibiotics, that may provide an alternative battle strategy against the growing public problem of antibiotic resistance (Shai 2002; Jiang et al. 2008; Huang et al. 2010). Despite their great potential as a novel class of antibiotics, their practical application in clinic has been hampered by some serious drawbacks. Their relatively long sequences require high production cost. Their poor selectivity between prokaryotic and eukaryotic cells causes toxicity. Most importantly, their vulnerability to proteolytic enzymes results in poor bioavailability (Marr et al. 2006). Therefore, various strategies have been studied to complement those weaknesses of AMPs.

In our previous studies, we demonstrated that Verdine's all-hydrocarbon stapling system (Schafmeister et al. 2000; Kim and Verdine 2009; Kim et al. 2011; Verdine and Hilinski 2012) is a highly useful tool for the development of such short, selective, and metabolically stable AMPs. As part of such effort, we reported a series of stapled heptapeptides as potential AMPs (Dinh et al. 2014, 2015; Luong et al. 2016, 2017). By virtue of the powerful helix-stabilizing effect of oct-4-enyl staple, these heptapeptides were designed to adopt amphipathic α-helices that have a hydrophilic face formed by three lysine residues at positions 1, 4, and 7 and a hydrophobic face formed by the oct-4-enyl hydrocarbon staple on the opposite side (Fig. 1). These stapled heptapeptides exhibited significantly enhanced stability against proteolytic degradation due to their conformational rigidity. They also displayed reasonable antimicrobial activity against both Gram-positive and -negative species of bacteria along with very low hemolytic activity against human red blood cells. In our previous

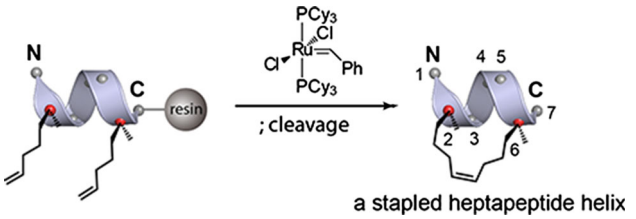

Fig. 1 Schematic presentation of the ruthenium-based all-hydrocarbon stapling chemistry that cross-links positions 2 and 6 via an oct-4-enyl tether yielding a stapled heptapeptide helix. Previous studies indicated that the $i,i+4$ stapling exclusively yields *cis* olefin (Bhattacharya et al. 2008; Zhang et al. 2008; Phillips et al. 2011)

study, we showed that the antimicrobial activity of this series is affected by various structural elements including the introduction of oct-4-enyl staple, position of tryptophan residue, net charge, property of *N*-terminal capping moiety, and chemical properties of residue at position 5, leading to peptide **KKK** as the most promising analog in this series (Luong et al. 2017) (Fig. 2a). However, its antimicrobial activity is not potent enough for clinical application and therefore has yet to be improved.

Cationic amino acids are important for the antimicrobial activity of most amphipathic helical AMPs because they mediate the initial interaction with negatively charged bacterial membranes via electrostatic attraction (Yeaman and Yount 2003; Lohner et al. 2008). In the sequence of peptide **KKK**, three lysine residues were also introduced for this purpose. However, it is well known that arginine is more effective in mediating such peptide-membrane interactions due to the stronger H-bonding capability of its guanidinium moiety compared to the primary amine moiety of lysine (Nguyen et al. 2011; Schmidt and Wong 2013; Li et al. 2013; Arias et al. 2018). Therefore, in this study we investigated the possibility of further improving antimicrobial activity of peptide **KKK** by substituting each lysine residue at position 1, 4, and 7 with arginine (Fig. 2b).

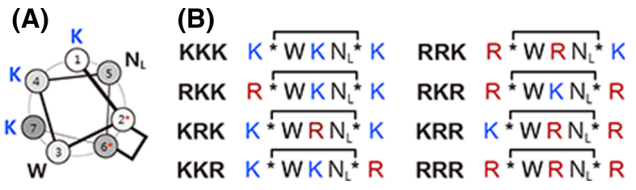

Fig. 2 **a** Helical wheel projection of the stapled heptapeptide **KKK**. **b** Sequences of mutated amphipathic heptapeptides studied in this report. The solid line connecting positions 2 and 6 represents the oct-4-enyl hydrocarbon staple. Asterisks in position 2 and 6 represent cross-linked residues. K, N_L, R, and W represent lysine, norleucine, arginine, and tryptophan residue, respectively. *N*-termini of all the peptides are in their free form and their *C*-termini are amidated

Materials and methods

General

Fmoc-(*S*)-α-methyl,α-petenylglycine-OH was purchased from Okeanos Tech Co. Ltd (Beijing, China). All other Fmoc-protected α-amino acids, Rink Amide MBHA resin, and 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino)]uranium hexafluorophosphate (COMU) were purchased from NovaBiochem (San Diago, CA, USA). Dimethylformamide (DMF), dichloromethane (DCM), piperidine, Grubbs 1st generation catalyst (bis(tricyclohexylphosphine)benzylidine ruthenium (IV) dichloride), *N,N*-diisopropylethylamine (DIEA), triisopropylsilane (TIS), *N*-methyl-2-pyrrolidinone (NMP), trifluoroacetic acid (TFA), and 1,2-dichloroethane (DCE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the commercially available reagents and solvents were used as received.

Peptide synthesis

Peptides were prepared using the published protocol (Kim et al. 2011). Briefly, Rink Amide MBHA resin (loading capacity = 0.6 mmol/g) was swelled in NMP for 10 min. The Fmoc group was eliminated using 25% piperidine in NMP (2 × 10 min). Peptide elongation was performed by treating the resin with a mixture of Fmoc-protected amino acid (5 equiv.), COMU (4.75 equiv.), and DIEA (10 equiv.) in NMP for 30 min. For the coupling of Fmoc-(*S*)-α-methyl,α-petenylglycine, 3 equiv. of Fmoc-protected amino acid was treated with COMU (2.85 equiv.) and DIEA (6 equiv.) for 2 h. The resin was washed with DCM (1 × 2 min), NMP (1 × 2 min), DCM (1 × 2 min), and NMP (1 × 2 min) after each coupling or deprotection reaction.

Metathesis and purification

Ring-closing metathesis (RCM) of protected peptides was performed on the solid support using 20 mol% of Grubbs I catalyst in degassed DCE at room temperature for 2 h. The RCM reaction was repeated using a fresh solution of Grubbs I catalyst for 2 h. After the resin was washed with DCE (3 × 2 min) and DCM (3 × 2 min), the Fmoc group was removed by treating with 25% piperidine in NMP (2 × 10 min). The resin was washed with DCM (3 × 2 min) and DMF (3 × 2 min) and dried *in vacuo* overnight. The peptides were cleaved from the resin by treating with a mixture of TFA/TIS/water (95/2.5/2.5) for 2 h. The products were precipitated by adding a mixture of *n*-pentane and diethyl ether (1:1). The mixture was centrifuged, and

the solvents was carefully decanted from the container to leave a mixture of the peptide products and the resin. After air-dried in 10 min, the peptides were dissolved in 50% acetonitrile in water. After the resin was filtered off, the peptide products were purified by reverse phase HPLC using a Zorbax C18 column (Agilent, 5 μm, 9.4 × 250 mm). The purified peptides were characterized using LC/MS (Shimadzu LCMS-2020).

KKK ESIMS *m/z* for $C_{47}H_{78}N_{12}O_7$ $[M+2H]^{2+}/2$ calcd 476.33, found 476.55

RKK ESIMS *m/z* for $C_{47}H_{78}N_{14}O_7$ $[M+2H]^{2+}/2$ calcd 490.33, found 490.35

KRK ESIMS *m/z* for $C_{47}H_{78}N_{14}O_7$ $[M+2H]^{2+}/2$ calcd 490.33, found 490.60

KKR ESIMS *m/z* for $C_{47}H_{78}N_{14}O_7$ $[M+2H]^{2+}/2$ calcd 490.33, found 490.50

RRK ESIMS *m/z* for $C_{47}H_{78}N_{16}O_7$ $[M+2H]^{2+}/2$ calcd 504.34, found 504.60

RKR ESIMS *m/z* for $C_{47}H_{78}N_{16}O_7$ $[M+2H]^{2+}/2$ calcd 504.34, found 504.55

KRR ESIMS *m/z* for $C_{47}H_{78}N_{16}O_7$ $[M+2H]^{2+}/2$ calcd 504.34, found 504.60

RRR ESIMS *m/z* for $C_{47}H_{78}N_{18}O_7$ $[M+2H]^{2+}/2$ calcd 518.34, found 518.60

GLU ESIMS *m/z* for $C_{46}H_{74}N_{12}O_9$ $[M+2H]^{2+}/2$ calcd 484.31, found 484.60

Circular dichroism

The sample solutions were prepared by dissolving each peptide in a 25 mM potassium phosphate buffer (pH 6.5). Peptide concentrations were determined based on the absorbance of tryptophan residue at 280 mm (λ_{280} = 5690 cm^{-1}). Circular dichroism (CD) spectra were collected on a Chirascan HP dual polarization CD spectrometer using standard measurement parameters: 1 nm bandwidth, 0.1 cm path length, 3 accumulations, 1 nm step resolution, and 0.5 s response. The CD spectra were converted into a uniform scale of molar ellipticity after background subtraction. The CD curves were smoothed using standard parameters.

Antimicrobial assay

Antimicrobial activity was determined by measuring the minimal inhibitory concentration (MIC) values using the standard broth microdilution method (Jorgensen and Ferraro 1998) with slight modifications. The assay was conducted against three Gram-positive strains (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538p, *Staphylococcus epidermis* ATCC 12228) and five Gram-negative strains (*Escherichia coli* ATCC 25922, *Shigella dysentariae* ATCC 9752, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumonia* ATCC 10031, *Pseudomonas aeruginosa* ATCC 27853). The bacteria were incubated in 2 ml Luria–Bertani broth at 37 °C overnight. The peptide solutions, prepared with twofold dilutions from 0.2 to 200 μg/mL, were mixed with the bacterial inoculums (10^6–10^8 colony-forming units (CFU)/mL) in 96-well round-bottom microtiter plates. After 24 h of incubation at 37 °C, each well was examined to determine MICs, the lowest peptide concentrations completely inhibiting cell growth. All the peptides were tested in duplicate. The bacterial strains used in this work were obtained from the Korean Collection for Type Culture at the Korean Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

Hemolysis assay

190 μL of human red blood cell suspensions (10% v/v in PBS) were treated with 10 μl of serially diluted peptides (ranging 0.8–200 μg/ml in PBS) and incubated at 37 °C for 30 min. After collected by centrifugation, the supernatants were diluted by tenfold with PBS and transferred to a new microtiter plate for spectrophotometric readings at 405 nm. 100% hemolysis was determined by treating the blood suspension with 0.2% Triton X-100. % Hemolysis was calculated based on the following equation:

$$\%\text{Hemolysis} = \frac{\text{OD}_{405\,\text{nm}}\ \text{sample}}{\text{OD}_{405\,\text{nm}}\ \text{positive control}}.$$

Results

To investigate effects of lysine-to-arginine substitution on the antimicrobial activity of **KKK**, we prepared seven mutant analogs using a previously reported protocol (Kim et al. 2011) (Fig. 2b). Mono-substitution at each position of 1, 4, and 7 of **KKK** yielded analogs **RKK**, **KRK**, and **KKR**, respectively. Double substitution at positions 1 and 4, 1 and 7, and 4 and 7 resulted in analogs **KRR**, **RKR**, and **RRK**, respectively. Substitution of all lysine residues of **KKK** with arginine gave rise to analog **RRR**.

The secondary structures of the peptide analogs were determined using far ultra-violet circular dichroism (CD) spectroscopy. All analogs displayed similar CD spectra to that of **KKK**, having two minima near 208 and 222 nm and a maximum near 190 nm, which are characteristic of α-helices (Fig. 3). Judging by the signal intensity at 222 nm (Chen et al. 1972), the parent **KKK** appeared to be the most helical among the peptides in this panel; all the arginine-substituted analogs were slightly less helical with **RRR** being the least helical.

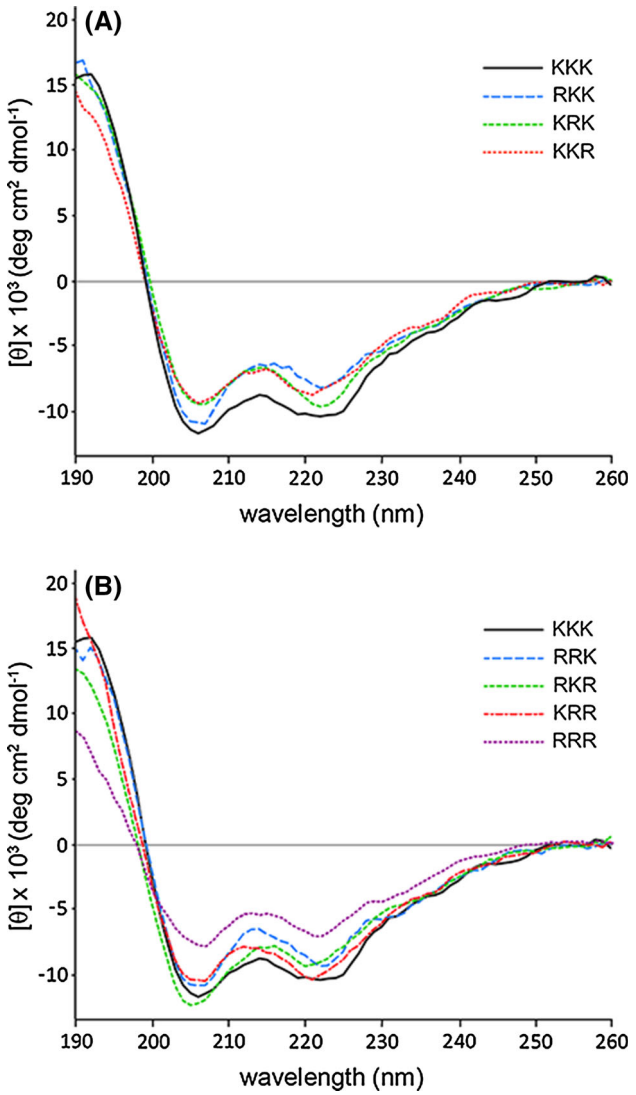

Fig. 3 Circular dichroism spectra of stapled heptapeptides in a 25 mM potassium phosphate buffer solution at 20 °C

The antimicrobial activities of the mutant peptides were evaluated in vitro against a set of representative bacterial strains including three Gram-positive and five Gram-negative species, using the broth microdilution method previously reported (Table 1). Analog **kkk**, which is a **KKK** derivative bearing glutamate in place of norleucine, was also included as a negative control in this assay (Luong et al. 2017). Compared to **KKK**, analogs **RKK**, **KRR**, and **RKR** displayed roughly twofold higher inhibitory activity against Gram-positive strains and a similar or twofold higher activity against Gram-negative strains. Analog **KKR** displayed an activity profile similar to **KKK** against both Gram-positive and –negative pathogens. In contrast, compared to **KKK**, analogs **RRK**, **RKR**, and **RRR** exhibited an approximately twofold decrease in activity against Gram-negative microbes, although their potency against Gram-positive microbes remained the same. All mutated peptides showed very low hemolytic activity against human red blood cells. Only **RKK**, **KRR**, and **RKR** displayed a slight increase at the given concentrations.

Discussion

Interactions with bacterial membranes are known to be a critical element for the antimicrobial action of most AMPs. Positively charged amino acid residues play a key role during the initial step of complex formation between AMPs and bacterial membranes due to their favorable electrostatic interactions with bacterial membranes. We previously reported a series of amphipathic helices of stapled heptapeptides containing lysine as the sole cationic amino acid residues, which showed reasonable antimicrobial activity against various bacterial species. In an effort to improve pharmacological properties of this series of novel AMPs, we examined the impact of lysine-to-arginine substitution on their structures and antimicrobial and hemolytic activities. We selected analog **KKK** as the parent control as it was the most active analog in the series (Luong et al. 2017) and systematically substituted its lysine residues with arginine, which yielded seven mutated analogs (Fig. 2).

Positively charged moieties in the side-chain of lysine and arginine residues have different chemical nature. Therefore, the lysine-to-arginine substitution introduced in the sequence of **KKK** may influence its secondary structures. In the CD analysis, all mutated analogs displayed a slight decrease in helicity compared to the parent **KKK** (Fig. 3). In particular, **RRR** was the least helical analog in this panel. This result indicated that lysine-to-arginine substitution has a negative influence on the helical structure of this series of stapled heptapeptides. Three cationic residues at position 1, 4, and 7 would reside on the same face of the helical scaffold formed by the helix-stabilizing oct-4-enyl staple. For this reason, all the stapled heptapeptides would suffer from a strong electrostatic repulsion in such a highly compact space. Arginine has a shorter and more basic side-chain compared to lysine (pK_a of the guanidino group of arginine is 12–13.7; the amino group of lysine, ~ 10.5). Therefore, electrostatic repulsion might be more severe when lysine residues are replaced with arginine. Unfavorable torsional strains induced by this stronger electrostatic repulsion in arginine-substituted analogs may be attributed to their slightly decreased helicity. This is most likely why peptide **RRR** has the lowest helical content in this series of stapled heptapeptides. Nonetheless, due to the strong helix-stabilizing capability of hydrocarbon staple, all mutated analogs still

Table 1 Antimicrobial MIC values of peptide analogs against selected bacteria and their hemolytic activity against human blood cells

Entry/code		Gram (+)			Gram (−)					Hemolysis, %[a]	
		B.s.	*S.a.*	*S.e.*	*E.c.*	*S.d.*	*S.t.*	*K.p.*	*P.a.*	50 μM	25 μM
1	**KKK**	12.5	6.3	25	12.5	25	50	18.8	25	3.0	1.5
2	**RKK**	6.3	6.3	12.5	12.5	25	25	12.5	25	4.3	1.5
3	**KRK**	12.5	6.3	25	25	50	75	37.5	25	2.0	< 1
4	**KKR**	9.4	6.3	25	12.5	25	50	12.5	25	5.4	1.4
5	**RRK**	12.5	6.3	25	25	37.5	100	50	37.5	< 1	< 1
6	**RKR**	6.3	3.2	12.5	6.3	25	25	18.8	25	17.0	8.2
7	**KRR**	6.3	3.2	12.5	12.5	25	50	18.8	37.5	5.6	1.8
8	**RRR**	12.5	6.3	25	25	50	100	50	100	3.2	< 1
10	**kkk**[b]	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	< 1	< 1

B.s. Bacillus subtilis, *S.a. Staphylococcus aureus*, *S.e. Staphylococcus epidermidis*, *E.c. Escherichia coli*; *S.d. Shigella dysenteriae*, *S.t. Salmonella typhimurium*, *K.p. Klebsiella pneumoniae*, *P.a. Pseudomonas aeruginosa*

Minimum inhibitory concentration is defined as the lowest peptide concentration (μM) that completely inhibits the cell growth after 24 h of incubation at 37 °C. The experiment was performed in duplicate

[a]Percent hemolysis is relative to that by 0.1% Triton X-100. The experiment was performed in duplicate

[b]Control peptide **kkk** is a **KKK** analog, reported in our previous study (Luong et al. 2017), containing glutamate in place of norleucine at position 5

appear to maintain α-helical conformations as the CD data revealed.

Helical content is well known as one of the critical determinants for the antimicrobial activity of many amphipathic helical AMPs (Huang et al. 2010). In our previous study, we demonstrated that helix-stabilization of the heptapeptides via an oct-4-enyl staple significantly promoted their antimicrobial activity. In contrast, their unstapled counterparts were shown to be unstructured and completely inactive against all the bacterial strains tested in the study (Dinh et al. 2014). In this current work, the helical content of the mutated peptides was not directly correlated with the antimicrobial activity; some arginine-substituted analogs showed an increase in inhibitory potency despite a slight decrease in helicity. Overall, lysine-to-arginine substitution in the sequence of **KKK** did not guarantee the global improvement in inhibitory activity against all bacterial strains tested in this work. Instead, a slight decrease in activity against Gram-negative microbes was observed for some arginine-substituted analogs (**KRK**, **RRK**, and **RRR**); the sensitivity of Gram-positive pathogens to these analogs remained the same as that of **KKK**. Also, some mutated peptides displayed a noticeable increase in activity against Gram-positive strains (**RKK**, **RKR**, and **KRR**). Their activity against Gram-negative bacteria did not significantly change compared to **KKK**. Interestingly, peptides that showed increased activity against Gram-positive strains also exhibited an increase in hemolytic activity compared to **KKK**.

In many cases, lysine-to-arginine substitution in natural AMPs gave rise to an increase in antimicrobial activity due to the more favorable interaction of guanidinium moiety of arginine with membranes. In this study, we examined the possibility of improving antimicrobial activity of **KKK**, the most active analog among our stapled heptapeptide series, by substituting lysine residues with arginine. Our results indicated that the lysine-to-arginine substitution does not always guarantee enhancement in antimicrobial potency of AMPs. Instead, we observed varied potency and selectivity depending on the number of the substitutions and which positions were substituted. Our results imply that, in the given helical scaffold stabilized by a hydrocarbon staple, antimicrobial potency and selectivity are influenced by a complex effect of various structural and chemical changes accompanied by lysine-to-arginine substitution rather than solely by the type of cationic residue. These data show potential for use in our scaffold-assisted development of short, selective, and metabolically stable AMPs.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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