PCR-based site-specific mutagenesis of peptide antibiotics FALL-39 and its biologic activities[1]

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KEY WORDS FALL 39; peptide antibiotics; polymerase chain reaction; mutation; hemolysis

ABSTRACT

AIM: To construct PGEX-1λT-FALL-39 expression vector and its mutant vector, and study the relationship of function and structure. **METHODS**: A cDNA encoding mature FALL-39 was cloned from SPCA-1 cell mRNA and the prokaryotic expression vector PGEX-1λT-FALL-39 was constructed. Two kinds of polymerase chain reaction (PCR) for the site-direction mutagenesis were used to construct FALL-39 mutant expression vector, FALL-39-Lys-32 and FALL-39-Lys-24. Minimal effective concentration, minimal inhibitory concentration, and minimal bactericidal concentration were used to assay the antibacterial activities of these peptides. Effects of different solution on the antibacterial activity of FALL-39 and FALL-39-Lys-32 were observed by CFU determination. The hemolytic effects of these peptides were also examined on human red blood cells. **RESULTS:** Two site-specific mutants FALL-39-Lys-32 and FALL-39-Lys24 were obtained by PCR-induced mutagenesis. In comparison with two-step PCR which required two pairs of primers, one step PCR which required one pair of primers is a simple and efficient method for the PCR based site-specific mutagenesis. Using the prokaryotic expression system, the *E coli*-based products of recombinant FALL39 and its mutant peptides were also obtained. The antibacterial assay showed that FALL-39-Lys-32 and FALL-39-Lys24 were more potential in the antibacterial activity against *E coli* ML35p and *Pseudomonas aeruginosa* ATCC27853 than that of FALL-39, and no increase in hemolysis was observed at the antibacterial concentrations. The antibacterial activity of FALL-39-Lys-32 against *E coli* was more potent than that of FALL-39 in NaCl-containing LB medium, while its activity was almost the same as FALL-39 in SO_4^{2-} containing Medium E. **CONCLUSION:** PCR-based mutagensis is a useful model system for studying the structure and function relationship of antimicrobial peptides. Keeping α-helical conformation of FALL-39 and increasing net positive charge can increase the antibacterial activity of FALL-39 without increasing hemolysis at the antibacterial concentrations.

INTRODUCTION

Cathelicidins, a kind of peptide antibiotics, contain a highly conserved signal sequence and pro-region (cathelin) but show substantial heterogeneity in the C-terminal domain that encodes the mature peptide[1-3]. The only human cathelicidin isolated from human bone marrow was designated FALL-39 after the first four amino acid residues[4]. LL-37 (two-amino-truncated form of FALL-39) which is a cystein-free peptide that can adopt an amphipathic α-helical conformation is considered to

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play an important role in the first line of host defense against local infection and systemic invasion of pathogens at sites of inflammation and wounds[5,6]. In order to obtain this peptide antibiotics, we construct a prokaryotic expression system, pGEX-1λT-FALL-39 expression vector.

Recent studies showed that LL-37 was much less potent than cathelicidins from other animals, such as sheep SMAP-29, rabbit CAP-18[7]. In order to increase its antibacterial activity, some LL-37 analogues were synthesized and their activities were observed[8]. In the present study, we created two mutants of FALL-39 by PCR-induced site-specific mutagenesis, prepared their *E coli*-based recombinant products, and examined their biological functions.

MATERIALS AND METHODS

Isolation the cDNA encoding mature FALL-39 and construction its expression vector pGEX-1λT-FALL-39 Total RNA was purified from human pulmonary gland epithelial cell line SPC-A-1 cells. RT-PCR was employed to synthesize DNA of FALL-39 using *Taq* DNA polymerase (from TAKARA) under the following cycling conditions: 30 s at 94 ºC, 30 s at 55 ºC and 45 s at 72 ºC for 30 cycles. The following primer pair was designed according to its gene information from genebank X96735 and used in RT-PCR. P_A: AACGGAT CCTTTGCCCTGCTG; P_B: CCAGGATCCGGCACAC-ACTAG. The PCR products and PGEX1λT were digested by restriction endonuclease (*BMH*1) , ligated with each other and then transformated into *E coli* JM109. The recombinant prokaryotic expression vector, named as pGEX-1λT-FALL-39, was successfully constructed, demonstrating by sequencing analysis.

Generation of site-specific mutant FALL-39-lys-32 and construction of its expression vector pGEX-1λT-FALL-39-lys-32 The site-specific mutagenesis was performed in a two-step PCR. Two sets of primers were designed according to the gene sequence of the FALL-39, and mismatch was introduced into P_2 for CTT to ATT substitution at position 32. The primers were P_1: AACGGATCCTTTGCCCTGCTG; P_2: GGGTACAAGCTT(ATT) CCGCAAAAAATCCTT; P_3: TTTTGCGG AAG(AAT) CTTGTACCC; P_4: CCAGGA TCC GGC ACA CAC TAG. The first step of PCR was carried out for 30 cycles using TAKARA *Taq* DNA polymerase under the following cycling conditions: 30 s at 94 ºC, 30 s at 55 ºC, and 30 s at 72 ºC. The reaction mixture was extracted by gel-extraction kit (from TAKARA). In the second PCR reaction, the fragment obtained from the first reaction was used as template. The reactions were carried out in the same conditions as the first one. The amplified fragments from the second PCR reaction which containing the site-specific mutation were purified using gel-extraction Kit prior to subcoloning into *E coli* JM109. The mutant prokaryotic expression vector, pGEX-1λT-FALL-39-lys-32 was constructed as mentioned above, and verified by endonuclease restriction and sequencing analysis.

Generation of site-specific mutant FALL-39-lys-24 and construction of its expression vector pGEX-1λT-FALL-39-lys-24 The site-specific Mutagenesis was performed in a one-step PCR. An opposite pair of primers was designed from the sequence encoding mature FALL-39, and a mismatch was introduced into P_6 for C to A substitution as elucidated in Fig 1. The primers were P_5: TTTTAAACTCTTTGC-CAATCTTCTC; P_6: GAATTGTCA(C→A)AGAGAATC AAGG. pGEX-1λT-FALL-39 was used as template and PCR was performed by using Polybest DNA polymerase (from TAKARA) in 30 cycles. The cycling conditions were as the following: 1 min at 94 ºC, 1 min at 55 ºC, and 5 min at 72 ºC. The DNA-fragments containing the site-mutation were purified by gel-extraction kit prior to Blunting Kination reaction. After linked encircledly, the amplified fragment was introduced into the *E coli* JM109. The pGEX-1λT-FALL-39-lys-24 expression vector was verified by PCR and sequencing analysis.

Isolation and purification of FALL-39, FALL-39-lys-32, and FALL-39-lys-24 from their construct-transformed *E coli* The transformed *E coli* carrying pGEX-1λT-FALL-39, pGEX-1λT-FALL-39-lys-32, and pGEX-1λT-FALL-39-lys-22 were cultured in LB medium for 12 h in the presence of IPTG (isopropyl β-*D*-

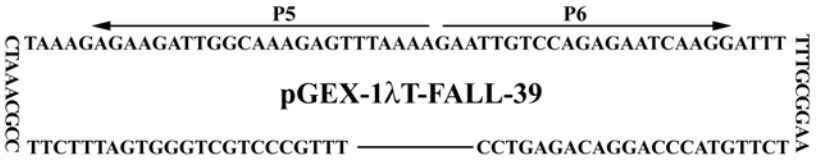

Fig 1. Creating FALL-39 site-specific mutant by one-step PCR.

thiogalactoside). The induced cultures were washed with PBS and the cell lysates were obtained by freezing/thawing in the presence of lysozyme. The fusion proteins were purified after centrifugation through Bulk Glutathione Sepharose 4B column (from Pharmacia) and monitored by SDS-PAGE. The purified fusion proteins were cleaved by thrombin and FALL-39, FALL-39-lys-32, and FALL-39-lys-24 were obtained by AU-PAGE elution.

Antibacterial activity assay

Evaluation of MEC The MEC (minimal effective concentration) were tested by a two-stage radial diffusion assay. Briefly, soy broth underlay gel mixture containing 1×10^6 colony-forming units (CFU)/mL of organisms was decanted into a dish. Sample wells of ϕ3 mm were punched and 5 μL peptides dilutions (100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/L) were added to the wells. After 3 h of incubation, overlay soy broth gels were poured and continuous incubated at 37 ºC overnight, the resulting clear zones were measured and expressed in units (1 mm=10 U) after subtracting the well diameter. A linear regression analysis of peptide concentration (*X* axis) against the zone diameter (*Y* axis) was performed so as to determine the *X* intercept, whose value represented the MEC.

Evaluation of MIC and MBC The minimal inhibitory concentration and minimal bactericidal concentration (MIC & MBC) of the peptides was examined in the soy broth containing bacteria at the concentration of 1×10^6 CFU/mL and the peptides in serial dilutions (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/L). Inhibition of growth was determined by measuring *OD* at 492 nm with UV/VIS spectrometer after incubation at 37 ºC for 12-16 h. Antimicrobial activity was expressed as the MIC, the concentration at which 100 % inhibition of growth was observed, and the MBC, the concentration at which no colony-forming units was observed after incubation for 12-16 h on soy broth solid medium.

Determination of effect of NaCl and SO_4^{2-} on antibacterial activity Effects of NaCl and SO_4^{2+} on antibacterial activity were determined[14]. *E coli* ML35p 1×10^6 CFU/mL were seeded in serial concentrations of FALL-39 and FALL-39-lys-32 containing media (Medium E, LB medium, and LB medium without NaCl) and incubated at 37 ºC for 12 h. Using CFU counting, bacterial growth was determined. Medium E (mmol/L): $MgSO_4$ 0.8, citric acid 9.6, K_2HPO_4 57.4, $NaNH_4HPO_4$ 16.7.

Testing hemolytic effects of peptides The hemolytic effects of the peptides was tested against human red blood cells (hRBC). Fresh hRBC were rinsed three times with PBS and resuspended in PBS in propriety concentration. Peptides, dissolved in PBS, were added to hRBC solution to the final concentration of 5 % (v/v). The resulting suspension was incubated at 37 ºC for 1 h. Release of hemoglobin was monitored by measuring the absorbance of the suspernatant at 540 nm after centrifugation. Controls for zero hemolysis (blank) and 100 % hemolysis consisted of hRBC suspended in PBS and hRBC suspended in 1 % Triton-X 100 PBS respectively. The hemolysis percentage was calculated by the following formula:

$$\text{Hemolysis}=[(A_{540\text{ nm in the peptide solution}}-A_{540\text{ nm in PBS}})/(A_{540\text{ nm in 1 \% Triton-X 100}}-A_{540\text{ in PBS}})]\times100\ \%$$

RESULTS

Isolation of the cDNA encoding mature FALL-39 and construction of its expression vector pGEX-1λT-FALL-39 A 146-bp cDNA encoding mature FALL-39 was obtained (Fig 2). Map of pGEX-1λT-FALL-39 was shown in Fig 3. Sequence analysis indicated that the insert sequence and its orientation were correct in the recombinant vector. The primary nucleotide and its deduced amino acid sequences were aligned in Fig 4.

Generation of site-specific mutants FALL-39-lys-24 and FALL-39-lys-32 and construction of their expression vectors pGEX-1λT-FALL-39-lys-24 and pGEX1λT-FALL-39-lys-32 Generating mutant pGEX-1λT-FALL-39-lys-32 was shown in Fig 5. The panel A

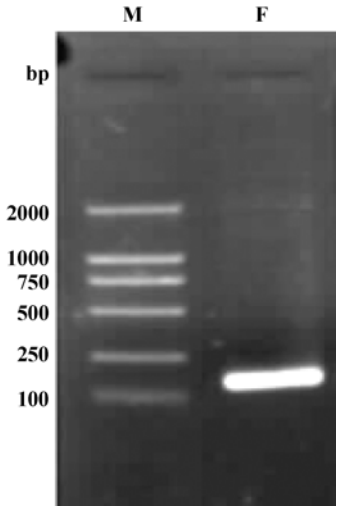

Fig 2. A cDNA encoding mature FALL-39. M: marker; F: cDNA fragment.

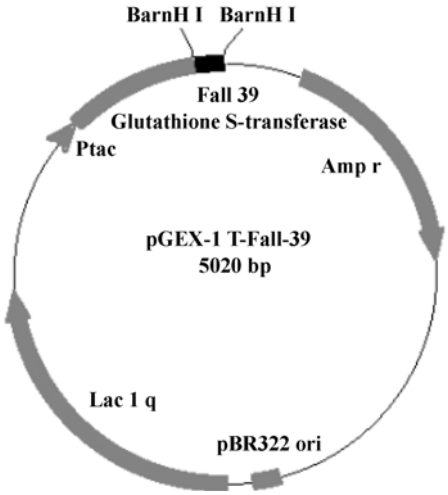

Fig 3. Map of pGEX-1λT-FALL-39.

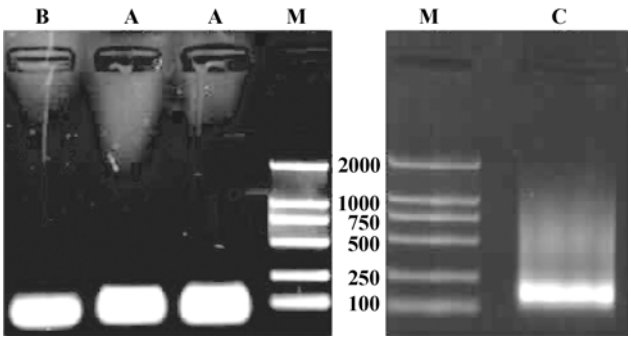

Fig 5. The fragments of FALL-39-lys32 DNA of PCR site-directed mutagenesis. A and B: the first PCR products; C: the second step PCR product; M: DNA marker.

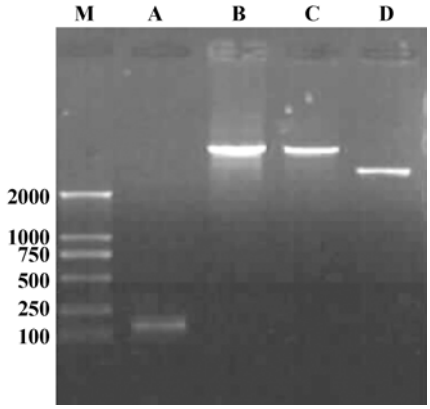

Fig 6. PCR and analysis of FALL-39-Lys24. A: PCR product from pGEX-1λT-FALL-39-lys24; B: mutant PCR product of the pGEX-1λT-FALL-39; C: *BMH*1-digested fragments of pGEX-1λT-FALL-39-lys24; D: pGEX-1λT- FALL-39-lys24; M: DNA marker.

and B were the products of the first PCR amplification which was 114 bp and 52 bp, respectively. The second step PCR produced a fragment with the size of 146 bp as shown in panel C. DNA sequencing verified this mutagenesis.

As shown in panel B of Fig 6, using one-step PCR amplification procedure, an about 5000 bp PCR product containing site-specific mutant FALL-39-lys-24 was obtained. After digested with *BMH* I, an around 140 bp fragment was released as shown in the panel C. DNA sequence analysis verified its sequence.

Deduced amino acid sequences of FALL-39 and its two site-specific mutants were aligned as in Tab 1. The most notable difference between FALL-39 and its pointed mutants is the increase in net charge.

Purification of peptides FALL-39, FALL-39-lys-32, and FALL-39-lys-24 The transformed *E coli* produced bulk amount of FALL-39, FALL-39-lys-32, and FALL-39-lys-24 fusion proteins. The molecular weight of fusion proteins was about 30 kDa as shown in Fig 7. The peptides FALL-39, FALL-39-lys-32, and FALL-39-lys-24 were obtained by AU-PAGE elution (Fig 8). It was clearly noted that FALL-39-lys-32 and FALL-39-lys-24 run faster than FALL-39, suggesting that the FALL-39 mutants had more positive net charge compared with native FALL-39.

Antibacterial activity

MIC, MEC, and MBC FALL-39-Lys-24 and FALL-39-lys-32 were more potent than FALL-39 against *E coli* ML-35p and *P aeruginosa* ATCC27853 (Tab 2).

Effect of NaCl and SO_4^{2-} on antibacterial activity As shown in Tab 3, antibacterial activity of FALL-39-Lys-32 against *E coli* was more potent than that of

TTT	GCC	CTG	CTG	GGT	GAT	TTC	TTC	CGG	AAA	TCT	AAA	GAG
F	A	L	L	G	D	F	F	R	K	S	K	E
AAG	ATT	GGC	AAA	GAG	TTT	AAA	AGA	ATT	GTC	CAG	AGA	ATC
K	I	G	K	E	F	K	R	I	V	Q	R	I
AAG	GAT	TTT	TTG	CGG	AAT	CTT	GTA	CCC	AGG	ACA	GAG	TCC
K	D	F	L	R	N	L	V	P	R	T	E	S

Fig 4. cDNA sequence encoding mature FALL-39 and its deduced amino acid sequences.

Tab 1. Amino acid sequences of the peptide used in this study.

Peptides	Amino acid sequences	Net charge
FALL-39	FALLGDFFRKSKEKIGKEFKRIVQRIKDFFRNLVPRTES	+6
FALL-39-lys32	FALLGDFFRKSKEKIGKEFKRIVQRIKDFFR***K***LVPRTES	+7
FALL-39-lys24	FALLGDFFRKSKEKIGKEFKRIV***K***RIKDFFRNLVPRTES	+7

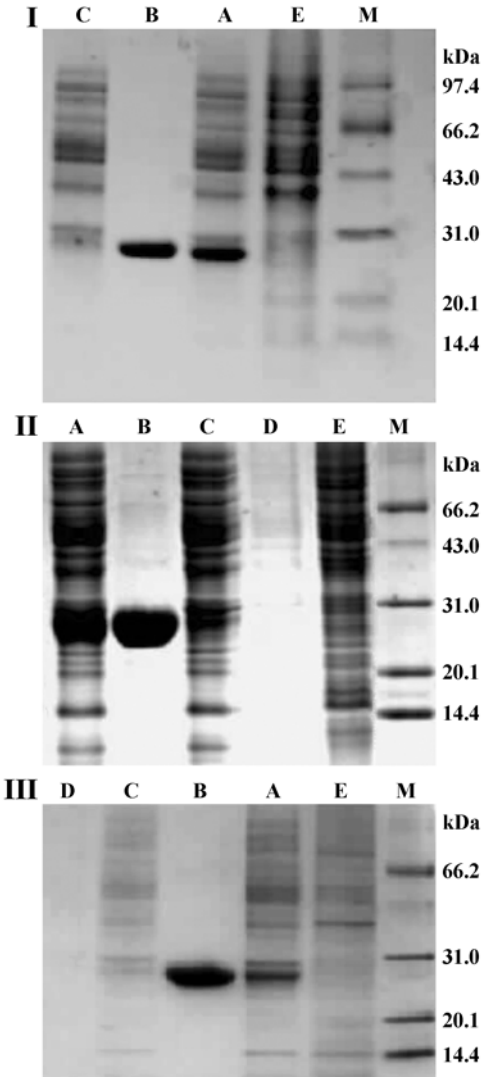

Fig 7. I: production of GST-FALL-39. II: production of GST-FALL-39-Lys-32. III: production of GST-FALL-39-Lys-24. A: IPTG-induced fusion protein; B: purified fusion protein; C: bacterial lysate supernatant; D: supernatant after washing by PBS; E: not induced by IPTG; M: protein marker.

FALL-39 in NaCl-containing LB medium. The antibacterial activity of FALL-39-Lys-32 was almost the same as FALL-39 in SO_4^{2-} containing Medium E, suggesting

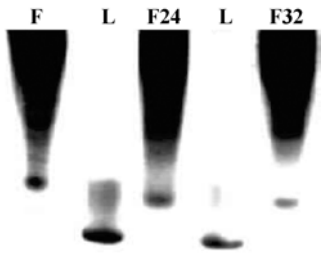

Fig 8. Thrombin-digested peptide fragments on AU-PAGE. F: FALL-39; F24: FALL-39-lys24; F32: FALL-39-lys32; L: lysozyme.

that the mutant remained amphipathic α-helical conformation[14].

Hemolytic effect FALL-39 and its mutants had some hemolytic effects on human red blood cells *in vitro* at very high concentration. However, there was no difference in the hemolytic effect between FALL-39 and its mutants FALL-39-lys32 and FALL-39-lys24 at antibacterial concentration (Fig 9).

Tab 2. Antibacterial activity of FALL-39 and its mutant peptides. *n*=4. $^{b}P<0.05$, $^{c}P<0.01$ *vs* FALL-39.

Strain of bacteria		*E coli* ATCC 25922	*E coli* ML35p	*P aeruginosa* ATCC 27853
FALL-39/mg·L^{-1}	MIC	19±3	19±37	31±3
	MBC	38±4	38±4	38±4
	MEC	NT	21±4	NT
FALL-39-Lys32/ mg·L^{-1}	MIC	9±4^{b}	11±3^{c}	19±3^{c}
	MBC	19±4^{b}	22±3^{c}	31±2
	MEC	NT	10±4	NT
FALL-39-Lys24/ mg·L^{-1}	MIC	9±4^{b}	10±3^{c}	21±7^{c}
	MBC	19±4^{b}	21±2^{c}	31±3
	MEC	NT	NT	NT

Note: NT means not tested.

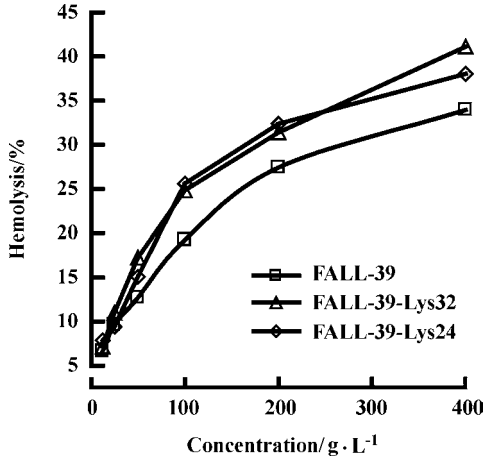

Fig 9. Effect of FALL-39, FALL-39-lys-32, and FALL-39-lys-24 on hemolysis of hRBC.

DISCUSSION

PCR-based site-specific mutagenesis Several methods for PCR-based site-specific mutagenesis have been described in the last few years[9-11]. The two-step PCR technique requires two pairs of primers. This procedure also requires two subsequent amplification rounds: the first amplification is carried out with two pairs of primers respectively to generate two fragments, and these purified fragments were used as template in the second amplification with a pair of universal primers. A possible problem in this technique is that one nucleotide would be intemplatedly added in the first amplified fragment by *Taq* polymerase, which could give rise to unwanted mutations in the second generated fragment. Recent studies suggest that the error frequency in the PCR would generate, on the average, after 30 cycles reaction, one base pair change in a 400 bp molecule. This technique is preferable to amplify small DNA segment[11] just as done in our experiment for creating the site-specific mutant FALL-39-lys32. Very recently improved method is the one-step PCR technique just as used in our experiment which requires a pair of opposite primers, one of them carrying a site-directed mutagenesis. This method is simple, and it can be used in almost all plasmids because of high fidelity and effectiveness of polybest DNA polymerase. Point mutations, deletion and replacements of small gene segments can easily be constructed. However, it must be noted that the primers used in this method should be opposite in direction. The DNA sequence of plasmid should be considered, otherwise the primers may combine with the plasmid nonspecificly.

Mutant of FALL-39 Recent studies showed that LL-37 was much less potent than cathelicidins from other animals, such as sheep SMAP-29, rabbit CAP-18. RL-37, an α-helical antimicrobial peptide of the rhesus monkey, was found to be more potent than LL-37 against *Staphylococci*[12]. The net charge of the peptide presumably is responsible for this event. Human LL-37 contains five acidic residues and has a net charge of +6, whereas rhesus RL-37 has only two acidic residues and a net charge of +8. In this study we created two mutants FALL-39-lys32 and FALL-39-lys24 that had an increase in net charge. As a consequence, their antibacterial activity was increased when compared with native FALL-39. The recent emergence of bacteria and fungi with resistance to well-known antibiotics, such as penicillin, ampicillin, gentamicin, even many new antibiotics, *etc*, has resulted in a considerable interest in developing the cationic α-helical antimicrobial peptides to be therapeutic antibiotics. Among the cationic α-helical antimicrobial peptides, FALL-39 was the only one in human beings. Pharmceutical application requires strong antibiotic activity against bacteria and fungi, the lower undesirable cytotoxic effect, and cheaper

Tab 3. Effect of FALL-39 and FALL-39-Lys32 on ML35p (10^{-6}×CFU/mL). $^{b}P<0.05$, $^{c}P<0.01$ *vs* FALL-39.

Dose/mg·L^{-1}	FALL-39			FALL-39-Lys-32		
	LB with Medium E	LB	LB without NaCl	LB with Medium E	LB	LB without NaCl
100	1.3±0.6	15.3±2.3	9.7±0.6	0±0	0±0^{c}	0.3±0.6^{c}
50	10.0±1.7	49.3±2.1	38±4	5.00±0^{b}	19.7±1.5^{c}	14.0±1.0^{c}
25	16.7±1.5	69±7	59±6	10.0±1.0^{b}	26.3±2.1^{c}	19.7±2.9^{c}
12.5	22±6	78±7	69±8	16±4	39.3±0.6^{c}	30.3±1.2^{c}
6.25	29±5	107.7±3.2	85±5	24±4	52±1.5^{c}	42.3±2.5^{c}
3.13	79.7±2.1	122.3±4	101±4	37±3^{c}	72±6^{c}	59.3±1.5^{c}

production. This study is approaching these goals.

Most mammalian antimicrobial peptides including defensins and cathelicidins are salt-sensitive. In this study, the created mutation of FALL-39 reduced its salt sensitivity, which would be favorable to maintain the antibacterial activity in body fluid. FALL-39 is consistent with a system in which largely unfolded monomeric peptide is in equilibrium with an α-helical oligomer form[13]. This is indeed the expected behavior of an amphipathic α-helical antimicrobial peptides. FALL-39 adopts an amphipathic α-helical conformation in a membrane-mimicking environment, a common motif in cell membrane lytic peptides. The driving force of oligomerization and concomitant α-helical formation is then expected to be due largely to the hydrophobic effect. Previous study has shown that some ions (SO_4^{2-}, HCO_3^-, $CF_3CO_2^-$ and to significantly lesser extent Cl^-) are favorable to helix formation in FALL-39[14]. Using these ions-containing media to study the antibacterial activity indicated that the mutant of FALL-39 with an increasing of net charges did not alter its α-helical formation.

Thus, the techniques used in this study may provide a useful model system for studying the function and structure relationship of mammalian antibiotic peptides, and for preparing the recombinant products of such antimicrobial peptides.

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