Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1109–1113

Development of novel lipid–peptide hybrid compounds with antibacterial activity from natural cationic antibacterial peptides

Hyun-Sik Oh, Seunghee Kim, Hyeongjin Cho and Keun-Hyeung Lee*

Department of Chemistry, Inha University, 253 Younghyong-Dong, Nam-Gu, Inchon-City, 402–751, South Korea

Received 1 December 2003; accepted 24 December 2003

Abstract—Seven depsipeptides were synthesized by appending seven amino acids (Lys, Leu, Val, Phe, Ser, Gln, and Pro) at the N-terminus of the active fragment [TE-(33-43)], respectively corresponding to the C-terminal β sheet domain of tenecin 1, an antibacterial protein and their activities were measured against *Staphylococcus aureus*. Considering the relationship between the activity and the characteristic of amino acid at the N-terminal of the peptide, novel derivatives were designed and synthesized from TE-(33-43) by introduction of fatty acids at the N-terminal. In this process, we synthesized novel lipid–peptide hybrid compounds with a potent antibacterial activity and more improved bioavailabilities. We characterized the important structural parameters of the lipid–peptide hybrid compounds for the antibacterial activities.

1. Introduction

The recent emergence of multi-drug resistant bacteria has demanded the development of novel antibacterial agents.[1] A large number of cationic antibacterial proteins and peptides have been identified from various natural sources.[2] Many analogues of natural antibacterial peptides were artificially synthesized for the development of novel antibacterial agents.[3] A number of studies revealed that most of antibacterial peptides exerted their activity by enhancing the permeability of pathogenic cell membranes and this kind of antibacterial peptides may be difficult to induce resistant strains of pathogens compared to classic antibacterial agents.[3,4] In the development process, the minimum active fragment was frequently identified from parent antibacterial peptides or proteins and then several analogues of the active fragment were synthesized by replacement, appending, and/or deletion of amino acids to enhance its spectrum, potency, and therapeutic index.[3] However, these antibacterial peptides themselves may not be used as therapeutic agents because of their poor in vivo stability and a risk of degradation by enzymes released from pathogens.

Interestingly, novel linear active fragments have been identified from cyclic antibacterial proteins containing disulfide bridges such as insect defense proteins and mammalian defense proteins by a random digestion with enzyme or chemicals and by the random synthesis of the truncated form of the native peptides.[5] Previously, we also successfully identified the active fragment from tenecin 1 (TE), an antibacterial protein, which belonged to the insect defensin family by synthesizing the peptides corresponding to the three regions of the protein.[6] Surprisingly, the fragment, [TE-(33-43)] corresponding to the C-terminal β sheet domain showed activity against fungi as well as Gram positive and negative bacteria while tenecin 1, a native protein showed activity only against Gram positive bacteria. However, the potency of the antibacterial activity of the fragment, [TE-(33-43)] was much lower than that of the parent protein. Moreover, considering the flexible and linear structure of the fragment compared to a compact and solid three-dimensional structure of the parent protein with three-disulfide bridges, the stability of the linear fragment was not guaranteed in vivo system.

In the present study, to improve antibacterial activity and stability of the active fragment, we developed novel derivatives of TE-(33-43) as follows. First, seven depsipeptides were synthesized by appending seven amino acids (Lys, Leu, Val, Phe, Ser, Gln, and Pro) at the N-terminus of TE-(33-43), respectively and their activities were measured against *Staphylococcus aureus*. Second,

Keywords: Antibacterial peptide; Structural parameters; Lipid peptide hybrid compound; Net positive charge; Stability.
* Corresponding author. Tel.: +82-32-860-7674; fax: +82-32-867-5604; e-mail: leekh@inha.ac.kr

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.12.085

considering the relationship between the activity and the characteristic of amino acid at the N-terminal, novel derivatives were designed and synthesized from TE-(33-43) by introduction of fatty acids or amino acids at the N-terminal. In this process, we developed novel lipid–peptide hybrid compounds with a potent antibacterial activity and more improved bioavailability. In addition, a simple method to improve the activity and the stability of linear cationic antibacterial peptide was developed and important structural parameters for the activity of cationic antibacterial peptides were characterized.

2. Synthesis

All tested peptide were synthesized by the Fmoc-chemistry in solid-phase peptide synthesis according to the literature procedure.[7] Fatty acid was introduced to the peptide by reaction of acetylated or Boc protected 6-aminocaproic acid and palmitoleic acid with the free amino terminal of the resin bound peptide, in the presence of DIC or pyBOP in *N*,*N*-dimethylformamide containing 1% DMAP. After cleavage of the product from resin, the peptides and lipid–peptide compounds were purified from crude product by preparative HPLC with a C_{18} column. The success of synthesis of compound was confirmed by ESI mass (Platform II, micromass, Manchester, UK) and the homogeneity (>95%) of the compound was confirmed by analytical HPLC with a C_{18} column.

3. Antimicrobial activity and hemolytic activity

Detail in vitro antimicrobial assay and hemolytic assay was described elsewhere.[8,9] Antibiotic medium 3 (M3; pH 7.0 at 25 °C, Difco) was used as antibacterial assay media. Freshly grown cells on antibiotic medium 3 agar plate were suspended in physiological saline to 10^4 cells per 1 mL of 2 X-concentrated medium and used as the inoculum. Peptide was added to the 96-wells (100 μL/well) in microplate and serially diluted by twofold. After inoculation, plates were incubated at 37 °C for 24 h and the absorbance at 620 nm was measured by ELISA reader (Spectra, SLT, Salzburg, Austria) to assess cell growth. Antifungal assay was done in Sabraud-2% dextrose broth (SB; pH 5.6 at 25 °C, Merck) and the plates were incubated at 30 °C for 24 h. Antimicrobial activity was expressed as the minimal inhibitory concentration (MIC), which was defined as the concentration at which 100% inhibition was observed. All MICs were determined from two independent experiments performed in duplicate. Magainin II and melittin were used as a reference compound in antimicrobial assay.

Mouse blood was centrifuged and packed mouse erythrocytes were washed three times with buffer. Various concentration of compounds were incubated with the erythrocyte suspension [final erythrocyte concentration of 1%] for 1 h 37 °C. And then after centrifugation at 4000×*g* for 5 min, the absorbance of the supernatant was measured at 540 nm and lysed erythrocytes by 0.1% Triton X-100 were considered as the standard for 100% hemolysis.

4. Compound stability in the presence of serum

Detail in vitro stability assay was described elsewhere.[10] After adding 10 μL of peptide stock solution (10 mg/mL) into1 mL of 25% mouse serum at 37 °C, the initial time was recorded and 100 μL of each reaction solution was removed at known time intervals and added into 100 μL of 10% aqueous trichloroacetic acid solution. The sample solution was cooled at 4 °C and spun to precipitate serum protein. Peptide analysis was carried out by reverse phase HPLC with Waters C_{18} column. Kinetic analysis for half-life of the sample was carried out by a linear least square analysis of the logarithm of the peak area versus time.

5. Results and discussion

We improved antibacterial activity of the fragment [TE-(33-43)] corresponding to the C-terminal β sheet domain of tenecin 1 as follows. First, various amino acid was appended at the N-terminal of the active fragment, respectively and the activity against *S. aureus* was investigated. As shown in Figure 1, seven amino acids (Lys, Leu, Val, Pro, Ser, Phe, and Gln) instead of twenty natural amino acids were selected for the synthesis of the derivatives of TE-(33-43) by consideration of the characteristics of amino acids.[11] One additional amino acid was introduced at the N-terminal of the peptide. Because β sheet structure of TE-(33-43) was prerequisite for the activity[6] and we expected that the introduction of only one amino acid at the N-terminal might not change the major secondary structure of TE-(33-43) and we could find out most important structural parameters except the secondary structure for antibacterial activity.

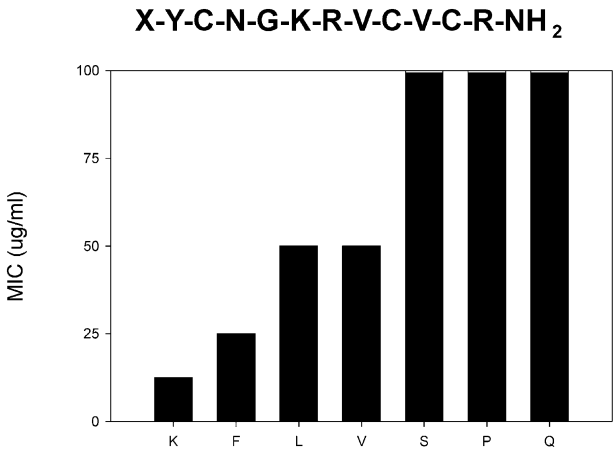

Figure 1. Screening of peptide mixtures synthesized by appending amino acid for activity to inhibit the growth of *S. aureus*. Each individual bar represented the minimal inhibition concentration (μg/mL) of peptide mixture defined in the 'X' position with one of the seven amino acid (K, F, L, V, S, P or Q).

Table 1. Sequence, net positive charges, retention time on C18 column and antimicrobial activities of TE-(33-43) and its analogues

Name	Sequence[a]	Net charge	CH_3CN (%)	Minimum inhibitory concentration (μg/mL)					HD_{50} (μg/mL)
				S. aureus ATCC 6538	*M. luteus* ATCC 9341	*P. aeruginosa* ATCC 9027	*E. coli* ATCC 25922	*C. albicans* ATCC 36232	
TE-(33-43)	YCNGKRVCVCR-NH_2	4	16	25	25	50	12.5	25	> 100
K-TE(33-43)	K-YCNGKRVCVCR-NH_2	5	16	12.5	6.3	50	50	25	> 100
F-TE(33-43)	F-YCNGKRVCVCR-NH_2	4	18	25	12.5	25	25	25	> 100
KK-TE(33-43)	KK-YCNGKRVCVCR-NH_2	6	17	6.3	6.3	25	25	12.5	> 100
KF-TE(33-43)	KF-YCNGKRVCVCR-NH_2	5	20	6.3	6.3	50	25	12.5	> 100
Ac-TE(33-43)	Ac-YCNGKRVCVCR-NH_2	3	20	50	100	NA	NA	NA	> 100
Bz-TE(33-43)	Bz-YCNGKRVCVCR-NH_2	3	24	100	> 100	NA	NA	NA	> 100
6AC-TE(33-43)	6AC-YCNGKRVCVCR-NH_2	4	21	50	50	NA	NA	NA	> 100
6AAC-TE(33-43)	6AAC-YCNGKRVCVCR-NH_2	3	22	> 100	> 100	NA	NA	NA	> 100
Pal-TE(33-43)	Pal-YCNGKRVCVCR-NH_2	3	55	> 100	> 100	NA	NA	NA	> 100
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS	4	NA	40	50	20	20	50	NA
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	6	NA	3.12	0.78	1.56	3.12	1.56	NA

[a] ac: acetic acid, Bz; Benzoic acid, 6AC: 6-aminocaproic acid, 6AAC: acetylation of 6-aminocaproic acid, Pal: palmitoleic acid, HD_{50} is the concentration of half-maximal lysis of mouse erythrocytes.

As shown in Figure 1, the most active peptide sequence had Lys at the N-terminal, which was consistent with our previous result where the net positive charge of TE-(33-43) was important for antibacterial activity.[6] When Phe was located at the N-terminal, the derivative showed the same MIC of TE-(33-43); however, the 50% inhibitory concentration was much lower when the Phe was located (data not shown). As shown in Table 1, each peptide analogue was synthesized by appending Lys and/or Phe residues and their activities were tested against bacteria and fungi. Generally, reverse phase-HPLC retention time was proportion to the real hydrophobicity of membrane-active peptides.[12] As shown in Table 1, the addition of two Lys residues or one Lys residue at the N-terminal decreased MIC value for Gram-positive bacteria four times and two times, respectively. The sequential addition of Phe and Lys residues at the N-terminal also improved the activity against Gram-positive bacteria four times and against fungi two times. These results strongly suggested that the net positive charge of the peptide was an important parameter for the activity against Gram-positive bacteria and fungi and the hydrophobicity also played a considerable role in the activity.

Considering the important parameters for the activity of TE-(33-43), we designed and synthesized novel lipid–peptide hybrid compounds from TE-(33-43) as shown in Figure 2. As shown in Table 1, the appending of palmitoleic acid and 6-acetylated aminocaproic acid at the N-terminal amino group of the peptide resulted in the loss of antibacterial activity while appending of 6-aminocaproic acid at the N-terminal amino group had decreased the activity just two times. When fatty acid was appending to the peptide, N-terminal amino group was blocked and the net positive charge of the peptide was decreased. To elucidate the important role of net positive charge at the N-terminal amino group, we synthesized N-terminal acetylated and benzylated of TE-(33-43) and measured antibacterial activity. As shown in Table 1, acetylation and benzylation of TE-(33-43) decreased antibacterial activity at least two times and four times, respectively, which revealed that the net positive charge of N-terminal was not critical but important for the antibacterial activity.

As shown in Table 1, TE-(33-43) and its analogues including lipid–peptide hybrid compounds were tested against erythrocytes. However, we could not clearly characterize the effect of fatty acid of the compound on hemolytic activity because all test compounds including lipid–peptide hybrid compounds did not show hemolytic activity. It seemed to be that introduction of fatty acid to the peptide must not increase hemolytic activity greatly.

Another purpose of introducing fatty acid to the peptide is to increase the resistance of the peptide against peptidase. When we compared the half life of TE-(33-43) and 6AC-TE(33-43), even though it was observed that the half life of 6AC-TE(33-43) was longer than that of TE-(33-43), TE(33-43) itself showed a considerable resistance against serum protease and the difference

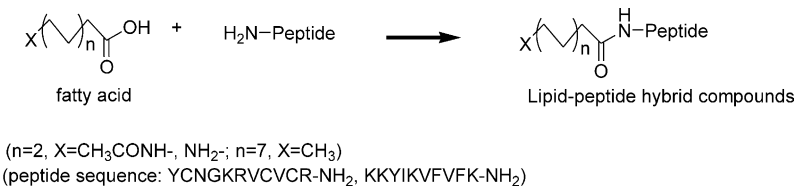

Figure 2. Schematic representations of lipid–peptide hybrid compounds with antibacterial activity.

Table 2. Stabilities and antibacterial activities of TE analogues

Name	Sequence	Minimum inhibitory concentration (μg/mL) for *S. aureus* ATCC 6538	Stability in the presence of serum (half-life, min)
TE analogue1	KKYIKVFVFK-NH_2	6.3	8
Ac-TE analogue1	Ac-KKYIKVFVFK-NH_2	100	30
Bz-TE analogue1	Bz-KKYIKVFVFK-NH_2	50	30
6AAC-TE analogue1	6AAC-KKYIKVFVFK-NH_2	6.3	45

between the half lives was less than two times (data not shown). As shown in Table 2, we synthesized a lipid–peptide hybrid compound by appending 6 amino caproic acid at the N-terminal of newly developed antibacterial peptide analogue of TE-(33-43). As shown in Table 2, this peptide which did not contain Cys residue and had higher net positive charge than TE(33-43), showed a more potent activity for *S. aureus* than TE(33-43). Interestingly, in this case, acetylation and benzylation of TE analogue1 decreased the activity fifteen times and eight times, respectively whereas the appending of 6-aminocaproic acid at the N-terminal of the peptide retained the antibacterial activity and increased the stability of the peptide in the presence of serum more than five times.

In this research, we showed an efficient method to characterize the structural parameters of antibacterial peptide by appending various amino acids to the N-terminal of the target peptide. In addition, we developed novel lipid–peptide hybrid compounds, which had a potent antibacterial activity and a more improved stability than the peptide.

Recently, two independent research groups reported the conjugation of long fatty acids to antibacterial peptides.[13] However, the conjugated fatty acids did not contain amino group and they did not report the antibacterial activity and stability of the conjugate. According to our result, the introduction of long fatty acid to the peptide resulted in the loss of antibacterial activity while introduction of amino fatty acid to the peptide retained the antibacterial activity. According to the retention time of lipid–peptide hybrid compounds, Pal-TE(33-43) was expected to have a potent antibacterial activity because the compound had the highest hydrophobicity in the series but this compound did not show antibacterial activity. The appending of long fatty acid, which did not contain amino group, decreased the net positive charge as an important parameter for the activity and/or lipid moiety in the hybrid compound may interfere the interactions between the peptide moiety in hybrid compound and lipid membranes of microorganisms. However, the appending of amino fatty acid retained net positive charge and lipid moiety may not interfere the interactions between the peptide moiety in hybrid compound and lipid membranes of target cells by charge–charge repulsions between amino group in lipid moiety and cationic peptide moiety. To elucidate the function of amino group in lipid moiety of the hybrid compound, we need a mechanism study and a secondary structure study of the lipid–peptide hybrid compounds in lipid membranes, which is now in progress. Considering the recent emergence of resistant bacteria, lipid–peptide hybrid compounds must be a new family of candidate for therapeutic agent against bacterial infection and the appending fatty acid to antibacterial peptides is a valuable method to develop antibacterial peptide analogues with a more improved bioavailability.

Acknowledgements

This work was supported by grant No.(R01-2001-000-00057-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

References and notes

1. (a) Travis, J. *Science* **1994**, *264*, 360. (b) Hata, K.; Kimura, J.; Miki, H. T.; Toyosawa, T.; Nakamura, T.; Katsu, K. *Antimicrob. Agents Chemother*. **1996**, *40*, 2237. (c) Sugawara, T.; Shibazaki, M.; Nakahara, H.; Suzuki, K. *J. Antibiot. (Tokyo)* **1996**, *49*, 345.
2. (a) Boman, H. G.; Hultmark, D. *Annu. Rev. Microbiol.* **1987**, *41*, 103. (b) Barra, D.; Simmanco, M. *Trends Biotechnol.* **1995**, *13*, 205. (c) Tossi, A.; Sandri, L. *Curr. Pharm. Design* **2002**, *8*, 743.
3. (a) Lee, K. H. *Curr. Pharm. Design* **2002**, *8*, 795. (b) Maloy, W. L.; Kari, U. P. *Biopolymers* **1995**, *37*, 105. (c) Sitaram, N.; Nagaraj, R. *Curr. Pharm. Design* **2002**, *8*, 727.
4. (a) Matsuzaki, K. *Biochim Biophys. Acta* **1999**, *1462*, 1. (b) Shia, Y. *Biochim Biophys. Acta* **1999**, *1462*, 55.
5. (a) Yamashita, T.; Yomogida, S.; Nagaoka, I.; Saito, K. *Biochem. Biophys. Acta* **1995**, *1243*, 295. (b) Hoek, K. S.;

Milen, J. M.; Grieve, P. A.; Donysius, D. A.; Smith, R. *Antimicrob. Agents Chemother*. **1997**, *41*, 54. (c) Little, G. R.; Kelner, D. N.; Lim, E.; Burke, D. J.; Conlon, P. *J. Biol. Chem*. **1994**, *269*, 1865. (d) Yamada, K.; Natori, S. *Biochem. J*. **1994**, *298*, 623.
6. Lee, K. H.; Hong, S. Y.; Oh, J. E.; Kwon, M. Y.; Yoon, J. H.; Lee, J. H.; Lee, B. L.; Moon, H. M. *Biochem. J*. **1998**, *334*, 99.
7. Fields, G. B.; Nobel, R. L. *Int. J. Pept. Protein Res*. **1990**, *35*, 161.
8. Lee, K. H.; Oh, J. E. *Bioorg. Med. Chem*. **2000**, *8*, 833.
9. Oh, J. E.; Hong, S. U.; Lee, K. H. *J. Pept. Res*. **1999**, *54*, 129.
10. Oh, J. E.; Lee, K. H. *Bioorg. Med. Chem*. **1999**, *7*, 2985.
11. Hong, S. U.; Oh, J. E.; Kwon, M. Y.; Choi, M. J.; Lee, J. H.; Lee, B. L.; Moon, H. M.; Lee, K. H. *Antimicrob. Agents Chemother*. **1998**, *42*, 2534.
12. (a) Krause, E.; Beyermann, M.; Dathe, M.; Rothemund, S.; Bienert, M. *Anal. Chem*. **1995**, *67*, 252. (b) Houghten, R. A.; DeGraw, S. T. *J. Chromatogr*. **1987**, *386*, 223.
13. (a) Avrahami, D.; Shai, Y. *Biochemistry* **2002**, *41*, 2254. (b) Eliassen, L. T.; Haug, B. E.; Berge, G.; Rekdal, O. *J. Pept. Sci*. **2003**, *9*, 510.