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Truncated and constrained helical analogs of antimicrobial esculentin-2EM

Thanh Kim Pham [a], Do-Hee Kim [b], Bong-Jin Lee [b], Young-Woo Kim [a,*]

[a] College of Pharmacy, Dongguk University, Seoul 100-715, Republic of Korea
[b] College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

Esculentin-2EM is a 37-residue, cationic, amphipathic, α-helical antimicrobial peptide isolated from a Korean frog, *Glandirama emeljanovi*. Many studies revealed that truncation of this peptide results in substantial decreases in its antimicrobial activity. Lee and his colleagues have recently reported that a 23-residue esculentin-2EM analog containing a tryptophanyl substitution at position 16 showed a significant recovery of the antimicrobial activity of the parent peptide. Here we report a new series of 15-residue esculentin-2EM analogs which are constrained into an α-helical conformation via an oct-4-enyl cross-link. The resulting 'stapled' derivatives displayed remarkable increases not only in antimicrobial activity but also in helical content and protease resistance compared to Lee's original 23-residue esculentin-2EM analog. The preliminary data obtained in this work strongly supports the potential of our strategy for the development of a new class of peptide antibiotics.

Antimicrobial peptides (AMPs) are naturally occurring antibiotics which exhibit potent broad spectrum antimicrobial activities and play an important role in the innate immune system in various organisms.[1–3] Because AMPs exert their antimicrobial activities via various distinctive modes of action, this specific class of molecules has recently emerged as a potential alternative to combat the growing problems of antibiotic-resistant bacteria.[4]

Amphipathic helical peptides are one of the most widely studied and well characterized classes of AMPs.[3,5–7] AMPs in this class have multiple cationic residues as well as multiple hydrophobic residues in their sequences. When interacting with bacterial membranes, they adopt an α-helical conformation, positioning the hydrophobic residues on one face of the helix and the hydrophilic residues on the opposite side. Both helicity and amphipathicity are considered as to be essential characteristics of this class of AMPs; the hydrophobic face is important for the interaction with the hydrophobic interior of the cell membrane, whereas the hydrophilic face plays a critical role in specific interactions with the membrane surfaces of certain microbes.[8–10]

Despite their promising potential as new antimicrobial therapeutics, the application of these amphipathic helical AMPs as therapeutics has been severely limited due to several undesirable properties.[11] For example, like many other peptide drugs, these AMPs are highly susceptible to proteolytic degradation and therefore suffer from a short *in vivo* half-life and poor bioavailability. Their inherent flexibility could also allow them to interact with various macromolecules besides the intended target, which could lead to undesired side-effects.[12] In addition, AMPs in this class are composed of more than 25 amino acid residues in many cases, which makes their preparation challenging and costly. For this reason, a great deal of research effort has been made to reduce the size of these peptides. However, in most cases the truncated analogs are accompanied by substantial decreases in antimicrobial activities.[13]

In this study, we investigated the possibility of improving the pharmacological properties of the amphipathic, helical AMPs using the emerging technology termed 'all-hydrocarbon peptide stapling'. This special cross-linking system is a powerful chemical tool that effectively stabilizes an α-helical conformation of a peptide by incorporating an all-hydrocarbon tether formed via ruthenium-mediated ring-closing olefin metathesis (Fig. 1).[17–19] Resulting

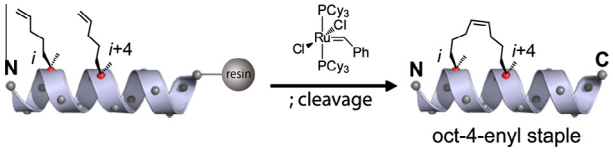

Figure 1. Schematic presentation of the all-hydrocarbon stapling chemistry tethering two residues at positions *i* and *i* + 4 via an oct-4-enyl tether. Elongation of peptide chains can be performed using a typical Fmoc/*t*-Bubased strategy on solid support. The fully-protected, resin-bound peptides are then subjected to ring-closing olefin metathesis using 20 mol % of Grubbs first generation catalyst in 1,2-dichloroethane at room temperature for 2 h. Previous studies indicated that the *i*,*i* + 4 stapling exclusively yields *cis* olefin.[14–16]

* Corresponding author.
E-mail address: ywkim730@dongguk.edu (Y.-W. Kim).

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'stapled' peptides were shown to have substantial increases in α-helical content, chemical and thermal stability, protease resistance, target binding affinity, and cell permeability.[20–26] We assumed that by effectively stabilizing the α-helical conformation using the peptide stapling system, a reasonable level of antimicrobial activity could be achieved from relatively short sequences of the known amphipathic, helical AMPs. To prove the potential of our strategy, we chose the sequence of esculentin-2EM,[27] a well studied antimicrobial peptide isolated from Korean frog *Glandirama emeljanovi*, as a model AMP in this study.

Esculentin-2EM is the longest member of the Gaegurin family, containing 37 amino acids. Like the other members of the Gaegurin family, esculentin-2EM is a cationic, amphipathic, α-helical peptide with a C-terminal disulfide bond and is known to exert antimicrobial activity by rupturing the bacterial membrane.[28–30] The helical conformation and amphipathic characteristic of esculentin-2EM are crucial for interacting with bacterial membranes and therefore also for its membrane-lytic activity. A great deal of research effort has been made to create esculentin-2EM analogs of a shorter sequence. However, truncating any residues of the esculentin-2EM sequence has always been accompanied by a substantial decrease in antimicrobial activity. For example, in 1999, Ryu and his colleagues reported that the N-terminal 23-residue fragment of esculentin-2EM showed a complete absence of antimicrobial activity.[31] This is most likely due to the lack of ability of the truncated derivative to form the biologically active α-helical conformation. In their recent studies, Lee and his colleagues demonstrated that substituting aspartic acid at position 16 with tryptophan (D16W) in the N-terminal 23-residue fragment of esculentin-2EM (**E2EM23W**) resulted in the recovery of helicity and, thus, the antimicrobial activity of esculentin-2EM as well.[32] It has been proposed that the tryptophan residue at position 16 in **E2EM23W** is located between the hydrophobic and hydrophilic faces of the amphipathic helix, and thus facilitate the amphipathic interactions between **E2EM23W** and the bacterial membrane surface. They also tested K15W and D16F substitutions in the N-terminal 23-residue fragment of esculentin-2EM, but neither substitution showed antimicrobial activities compatible with those of **E2EM23W**. Since **E2EM23W** represents the shortest analog of esculentin-2EM with reasonable antimicrobial activities identified thus far, we decided to explore our hypothesis through this molecule.

To compare the relative antimicrobial activities, we prepared **E2EM23W** as a positive control (Fig. 2). The main goal of the current study was to develop shorter and more potent analogs of this 23-residue control peptide. We also synthesized **E2EM15W**, a truncated analog consisting of 5–19 residues of **E2EM23W**, as a negative control (Fig. 2). Although these 15 residues are known to be involved in the core helical stretch of **E2EM23W** in SDS micelles,[32] we anticipated that **E2EM15W** would be less helical than the 23-residue **E2EM23W** mainly due to the absence of four residues at each of N- and C-terminus which would result in less potent antimicrobial activities.

	1	10	16	23
E2EM23W	Ac-GILDTLKQ	FAKGVGKW	LVKGAAQ	-NH_2
E2EM15W	Ac-TLKQ	FAKGVGKW	LVK	-NH_2
E2EM15W-S1	Ac-TLKQ	FXKGVXKW	LVK	-NH_2
E2EM15W-S2	Ac-TLKQ	FXKGWXKD	LVK	-NH_2
E2EM15W-S3	Ac-TLKQ	WXKGVXKD	LVK	-NH_2

Figure 2. Sequences of esculentin-2EM analogues. All the peptides were synthesized using a typical Fmoc/*t*-Bubased strategy on solid support. Cross-linking reactions were performed as described in Figure 1. X represents the residue cross-linked by an oct-4-enyl staple. Ac and NH_2 represent N-terminal acetylation and a C-terminal primary amide, respectively.

To examine the effects of all-hydrocarbon stapling on the antimicrobial activities, we prepared **E2EM15W-S1**. **E2EM15W-S1** is a stapled analog of **E2EM15W** with an oct-4-enyl staple that cross-links residues 10 and 14 in the sequence (Fig. 2). These two residues are located in the interface between the hydrophilic and hydrophobic faces.[30] Therefore, we rationalized that the introduction of a hydrocarbon staple between these two residues would minimize potential disruption of the amphipathic characteristic.

Installation of a hydrocarbon tether might cause a slight shift in the hydrophilic and hydrophobic faces of the helix. In a stapled scaffold, therefore, shifting the tryptophanyl substitution position may result in more favorable effects on the membrane-interacting property of the peptides. To examine this possibility, we also prepared **E2EM15W-S2** and **E2EM15W-S3**, which carried the tryptophan substitution at positions 9 and 13, respectively, instead of position 16 (Fig. 2). These two positions were chosen because of their physical proximity to the interface between hydrophilic and hydrophobic faces of the helix.

With a series of **E2EM** peptides in hand, we evaluated their antimicrobial activities against selected Gram-positive and Gram-negative bacteria (Table 1). Under the assay conditions used in this study, the 23-residue unstapled control, **E2EM23W**, showed minimal inhibitory concentrations (MICs, μg/mL) of 50 and 100 for *Bacillus subtilis* and *Staphylococcus aureus*, respectively, but no detectable activities against the other species including all of the Gram-negative bacteria. As expected, **E2EM15W**, the truncated analog of **E2EM23W**, was almost inactive in this assay. This is probably because this 15-residue analog is not long enough for the formation of an α-helix that would effectively disrupt bacterial membranes. To our satisfaction, all the stapled peptides exhibited significantly enhanced antimicrobial activities against these two species. **E2EM15W-S1** displayed the highest level of antimicrobial activities among the peptides tested in this study; its MICs were 3.13 for both *Bacillus subtilis* and *Staphylococcus aureus*. Since **E2EM15W-S1** is the stapled derivative of the inactive **E2EM15W**, these results clearly demonstrated the potential of the all-hydrocarbon stapling system to promote the antimicrobial activities of peptide antibiotics. Nevertheless, **E2EM15W-S1** did not show any detectable activities against *Staphylococcus epidermis*, a Gram-positive microbe, and all the Gram-negative species.

Although slightly less potent than **E2EM15W-S1**, the other stapled analogs **E2EM15W-S2** and **E2EM15W-S3** also displayed a notable increase in antimicrobial activities against *Bacillus subtilis* and *Staphylococcus aureus*. Unlike **E2EM15W-S1**, however, **E2EM15W-S2** and **E2EM15W-S3** exhibited considerably enhanced activities against some Gram-negative bacteria including *Shigella dysentariae* and *Klebsiella pneumonia* (Table 1). The sequences of the three stapled peptides in this series differ only in two amino acid residues, including tryptophan. Therefore, these results showed that the position of tryptophan substitution is a key element of the structure–activity relationships for this peptide series. Further systematic studies are required to more precisely elucidate the structure–activity relationships. Overall, the results obtained from this assay have clearly proved the potential of our approach not only to shorten the sequences, but also to modulate both the potency and specificity of certain antimicrobial peptides.

To test if the enhanced antimicrobial activities of the stapled analogs against some species are attributed to their helix stability, we examined their conformational preferences using the far ultraviolet circular dichroism spectra. In a previous study, **E2EM23W** was shown to exist as a random coil in an aqueous solution, but to adopt an α-helical conformation in a membrane-mimetic environment.[32] Because the all-hydrocarbon stapling system is an efficient chemical tool for re-enforcing a peptide into an α-helix via a covalent tether, the helicity of a stapled peptide is relatively independent from the environments.[14–18] Therefore, we analyzed the