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Influence of hydrocarbon-stapling on membrane interactions of synthetic antimicrobial peptides

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ABSTRACT

Cyclization has been recognized as a valuable technique for increasing the efficacy of small molecule and peptide therapeutics. Here we report the application of a hydrocarbon staple to a rationally-designed cationic antimicrobial peptide (CAP) that acquires increased membrane targeting and interaction *vs.* its linear counterpart. The previously-described CAP, 6K-F17 (KKKKKK-AAFAAWAAFAA-NH_2) was used as the backbone for incorporation of an *i* to *i* + 4 helical hydrocarbon staple through olefin ring closing metathesis. Stapled versions of 6K-F17 showed an increase in non-selective membrane interaction, where the staple itself enhances the degree of membrane interaction and rate of cell death while maintaining high potency against bacterial membranes. However, the higher averaged hydrophobicity imparted by the staple also significantly increases toxicity to mammalian cells. This deleterious effect is countered through stepwise reduction of the stapled 6K-F17's backbone hydrophobicity through polar amino acid substitutions. Circular dichroism assessment of secondary structure in various bacterial membrane mimetics reveals that a helical structure may improve – but is not an absolute requirement for – antimicrobial activity of 6K-F17. Further, phosphorus-31 static solid state NMR spectra revealed that both non-toxic stapled and linear peptides bind bacterial membranes in a similar manner that does not involve a detergent-like mechanism of lipid removal. The overall results suggest that the technique of hydrocarbon stapling can be readily applied to membrane-interactive CAPs to modulate how they interact and target biological membranes.

1. Introduction

Antimicrobial peptides have long been recognized as an excellent natural resource that may be exploited for the optimization and development of novel antibiotics.[1] These short peptides commonly range from 6 to 50 amino acids in size and occur with a wide array of compositions, sequences, and structures.[2] One of the largest categories is the cationic antimicrobial peptides (CAPs), which are rich in positively-charged amino acids such as Lys and Arg. These residues aid in targeting peptides selectively to the negatively-charged lipid head groups abundant in bacterial membranes upon which the high content of hydrophobic amino acids including large aromatics (*e.g.*, I, L, V, M, F, W) promote interaction with the hydrophobic interior of the lipid bilayer core. While various mechanisms of action have been proposed, generally CAPs function to disrupt or weaken the bacterial membrane, ultimately inducing cell death. Most natural CAPs function through the adoption of an α-helical structure that presents an amphipathic surface upon folding, with one helical face presenting hydrophobic amino acids to the membrane and the other presenting positively-charged amino acids to the adjacent aqueous medium.

We have previously designed and characterized the novel synthetic CAP 6K-F17 (KKKKKK-AAFAAWAAFAA-amide), in which the positively-charged residues are clustered at the *N*-terminus, thereby producing two separate domains of the peptide – one

Abbreviations: CAP, cationic antimicrobial peptide; TM, transmembrane; PPI, protein-protein interaction; Fmoc, Fluorenylmethyloxycarbonyl chloride; DMF, dimethylformamide; DCM, dichloromethane; DIEA, diisopropylethylamine; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; Pyclock, 6-chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; CD, circular dichroism; NMR, nuclear magnetic resonance; ssNMR, solid state nuclear magnetic resonance; MRE, mean residue ellipticity; SDS, sodium dodecylsulfate; MIC, Minimum Inhibitory Concentration; cfu, colony forming unit; MHC, Minimum Hemolytic Concentration; PBS, phosphate buffered saline; TI, therapeutic index; MHB, Mueller Hinton Broth; OD_{600}, optical density at 600 nm; CSA, chemical shift anisotropy; LUV, large unilamellar vesicle.

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0968-0896/

charged and the other an uninterrupted hydrophobic segment.[3] The unique design of 6K-F17 has been suggested to increase the functionality of this peptide over naturally occurring amphipathic CAPs by allowing the charge-separated uninterrupted hydrophobic domain to more deeply penetrate and disrupt the bacterial membrane in a 'grip and dip' mechanism.[3–5] 6K-F17 has been found to display high selectivity for bacterial membranes with concurrent low toxicity against mammalian cells, making it an excellent antimicrobial reagent.[3] In a strategy towards optimizing 6K-F17 as a therapeutic, we opted to cyclize the peptide to inhibit potential *in vivo* proteolytic degradation. Previously, 6K-F17 was cyclized through a side chain-side chain lactam bridge formed via the substitution of two of its Ala residues with an Asp and Lys near the termini of the peptide.[5] While the cyclized peptide could retain helical structure in membrane mimetics such as SDS micelles, there was no gain in antimicrobial activity; the effects on stability and toxicity to mammalian cells were not determined. We have now undertaken to introduce an all-hydrocarbon staple into 6K-F17 by cyclizing the peptide through incorporation during synthesis of two non-natural amino acids modified with olefin tethers in place of alpha-carbon protons (Fig. 1A). The hydrocarbon staple is then formed through an olefin ring closing metathesis reaction utilizing Grubbs catalyst.[6] The resulting 'stapled' peptide is anticipated to be locked into a helical conformation within the macrocyclic portion of the peptide, and to exhibit increased resistance to proteases as the susceptible peptide backbone becomes shielded (Fig. 1B, C).[7] The technique may be easily adapted to most common helical peptides and is a reaction that may be completed economically on solid phase resin supports.[6]

Hydrocarbon stapling through modified amino acid side chains using the Grubbs metathesis reaction was first applied to soluble peptide domains by Walensky *et al.* in 2004 for the disruption of soluble protein-protein interactions (PPIs).[8] While there has been much reported success in the use of hydrocarbon stapling of soluble peptides for membrane targeting and proteolytic protection,[9,10] its application to transmembrane (TM) domains and membrane-active peptides remains sparse. Previously we have successfully applied the hydrocarbon stapling technique to improve the metabolic stability of a TM peptide designed to inhibit PPIs within the lipid bilayer.[11] However, to our knowledge there are only limited reported examples of hydrocarbon stapling of antimicrobial peptides.[12–14] Of particular note, Dinh *et al.*, have applied single and double hydrocarbon staples to designed Ala-Lys peptides reporting improved antimicrobial activity; however, this procedure also increased toxicity compared to the parent unstapled peptides.[14] Here we expand the approach to 6K-F17 and analogs, and describe the first application of hydrocarbon stapling to this membrane-interactive antimicrobial peptide, and describe the accompanying structural and functional changes alongside toxicity against mammalian membranes.

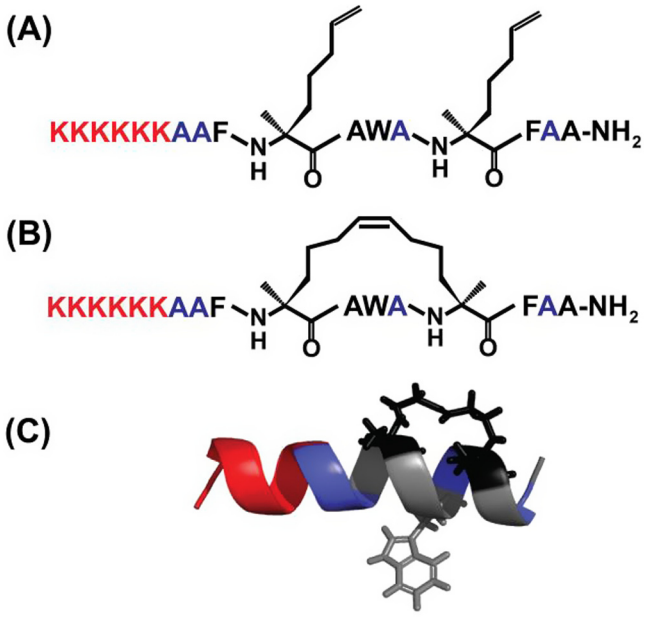

Fig. 1. Design of stapled cationic antimicrobial peptides. (A) Schematic of the primary sequence of S-6K-F17 with the 2-(4′-pentenyl)alanine olefin tethers replacing Ala-10 and Ala-14. (B) Ring closed depiction of hydrocarbon staple. (C) Structural model of S-6K-F17 as a helix with the added hydrocarbon staple at positions Ala-10 and Ala-14. The centrally located Trp residue side chain is included for reference in Trp fluorescence studies. The hydrocarbon staple and corresponding Ala residues are rendered in black. Positively-charged residues are depicted in red, neutral residues in grey, and positions for substitutions with Gly/Asn in blue.

2. Materials and methods

2.1. Peptide synthesis and purification

Linear peptide synthesis was automated using a PS3 peptide synthesizer (Protein Technologies, Inc.) using standard solid state Fmoc [N-(9-fluorenyl)methoxycarbonyl] chemistry on a low-load PAL–PEG resin (Applied Biosystems) that produced an amidated C-terminus after cleavage. Stapled peptide synthesis was automated except for the addition of staple precursor amino acids, (*S*)-*N*-Fmoc-2-(4′-pentenyl)alanine (Fmoc-S5Ala-OH) (Okeanos Technology Co., Beijing, China) as previously described.[11] Briefly, Fmoc-S5A-OH amino acids were hand coupled for 2 h in a 7:3 (v/v) mixture of DMF:DCM supplemented with Pyclock (Novabiochem, Canada) and DIEA. Amino acids following Fmoc-S5A-OH were double-coupled with HATU replaced with Pyclock. Olefin ring closing metathesis was performed on resin using 8 mg Grubbs 1st generation catalyst (Sigma-Aldrich, Canada) dissolved in 2 mL DCE and reacted for 2 h. The reaction was repeated twice to ensure full stapling. All peptides were purified using high- performance liquid chromatography (HPLC) with a C4 semipreparative column (250 × 21.20 mm, 300 Å pore size, Phenomenex, Canada). Typically, linear acetonitrile/water gradients (1% increase in acetonitrile per minute) were employed with initial conditions of 80% solvent A (95% water, 5% acetonitrile, and 0.1% TFA) and 20% solvent B (95% acetonitrile, 5% water, and 0.1% TFA). Peptides were quantified using the absorbance at 280 nm in water.

2.2. Liposome preparation

Total *E. coli* lipid extract (Avanti Polar Lipids Inc., Alabama, USA) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in chloroform (Avanti Polar Lipids Inc., Alabama, USA) was dried into thin films, lyophilized overnight and brought up in 1 mL water, vortexed, frozen and lyophilized again. The remaining lyophilized lipid was brought up in 10 mM Tris buffer 10 mM NaCl pH 7.4 (25 mg/mL) and freeze thawed 5×. Samples for CD and Trp fluorescence were extruded using a 0.2 μm sized filter and left to equilibrate overnight. Samples were then diluted appropriately for use (2 mg/mL for CD, 0.8 mg/mL for Trp fluorescence). Peptide was added to pre-formed liposomes and allowed to equilibrate overnight. Samples for ^{31}P static NMR were extruded using a 0.4 μm sized filter and left to equilibrate overnight. Peptide (250 or 500 μM) was added to pre-formed equilibrated liposomes (diluted to 12.5 mg/mL) immediately before spectra were taken.