Hydrocarbon stapled peptides: a new strategy to advance anti-microbial therapy

Review Article

New approaches are needed in the fight against antibiotic resistance. One is the use of short antimicrobial peptides that selectively disrupt the membranes of bacteria. A collaboration between the Hospital for Sick Children, Toronto, and the University of Toronto has led to new insights into how antimicrobial peptide activity can be modulated using stapling and substitution of residues. This is a promising approach in the development of new peptide therapeutics.

Antimicrobial peptides as new weapons against bacterial infection

Antibiotics are one of the greatest breakthroughs in modern medicine, but overuse and misuse has led to the rapid emergence of resistant bacteria worldwide (1). Added to that, poor return on investment and challenging regulatory requirements has hindered new drug development by the pharmaceutical industry. The result is that many bacteria are now serious threats, leading to substantial clinical and financial burdens on health care.

The development of resistance has stimulated the search for alternatives to conventional antibiotics. One natural source for the development of new weapons against microbes is antimicrobial peptides (2). These peptides are generally short (6–50 aa), with one of the largest categories being cationic antimicrobial peptides (CAPs). These peptides are rich in positively charged amino acids such as Lys and Arg and also have a hydrophobic region rich in large aromatics. The CAPs adopt an α-helix that folds to present the positively charged face to the aqueous medium while the hydrophobic face specifically targets the abundant negatively charged lipid head groups in bacterial membranes and weakens or disrupts the membrane to kill the bacterium.

Stapling a CAP to shield from protease attack

The authors (2) had previously designed a synthetic CAP, 6K-F17 (KKKKKK–AAFAAWAAFAA-amide), with clearly separated charged and hydrophobic domains. This CAP proved to be an excellent antimicrobial agent, with a high selectivity for bacterial membranes and low toxicity against mammalian cells. In an effort to further develop this peptide as a therapeutic, the authors cyclized 6K-F17 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, but this did not increase the antimicrobial activity.

In this study, the authors introduced an all-hydrocarbon staple into 6K-F17 by including two amino acids modified with olefin tethers in place of alpha-carbon protons and using these to cyclize the peptide through an olefin ring closing metathesis reaction utilizing Grubbs catalyst. They reasoned that the resulting stapled peptide would be locked into a helical conformation such that the peptide backbone is shielded from protease attack, which would improve the efficacy of the antimicrobial peptide.

Peptide design and synthesis

Staple precursor amino acids were placed near the center of the hydrophobic sequence, four residues apart to place the staple near the middle of the hydrophobic core on a single face of the folded helix (Figure 1). An 8-aa staple was chosen to optimize protease resistance.

Figure 1. The linear version of the CAP, 6K-F17, was stapled between Ala-10 and Ala-14 (underlined; S-6K-F17) and residues were substituted step-wise (bold).

Linear peptides were synthesized on a PS3® peptide synthesizer using standard solid phase Fmoc [N-(9-fluorenyl) methoxycarbonyl] chemistry on a low-load PAL_PEG resin that produced an amidated C-terminus after cleavage. Stapled peptide synthesis was automated except for the manual addition of staple precursor amino acids, (S)-N-Fmoc-2-(4-pentenyl)alanine (Fmoc-S5Ala-OH). Amino acids following Fmoc-S5A-OH were double-coupled with HATU replaced with PyClock. Olefin ring closing metathesis was performed on resin using Grubbs 1st generation catalyst. All peptides were purified using high-performance liquid chromatography (HPLC).

Antimicrobial activity is not dependent on helicity

The original peptide, 6K-F17, proved to be highly helical in a detergent micelle model and adding the staple increased the helicity slightly. Testing in a model comprised of *Escherichia coli* lipids, however, showed that the hydrocarbon-stapled S-6K-F17 peptide had the highest helicity of all the peptides. Introducing the polar 'helix-breaking' Gly and Asn amino acids reduced helicity, but despite these differences in helicity all versions of 6K-F17 had approximately the same antimicrobial activity.

Polar substitution improves selectivity after stapling

Adding the staple improved antimicrobial activity slightly but also greatly increased the toxicity towards mammalian cells. The increase in hydrophobicity by the hydrocarbon staple, increased non-specific membrane targeting, and thus decreased peptide selectivity for bacterial membranes. Increasing the peptide selectivity for bacterial membranes by replacing Ala^{8}, Ala^{13}, and Ala^{16} with Gly residues and Ala^{7} with an Asn residue, improved the toxicity profile.

Stapling helps embed the peptide into the micelle

The association of peptides with negatively charged micelles was measured using tryptophan fluorescence spectra in the presence and absence of detergent. The parent peptide, 6K-F17, was substantially associated with detergent micelles. Adding the staple in S-6K-F17 increased targeting and burial into the anionic micelle interior and this effect was reversed by the polar substitutions in the -2G, -3G and -3GN variants.

Stapling speeds up growth inhibition

Studies on cell growth showed that inhibition was concentration dependent and adding the staple resulted in more rapid growth inhibition. This effect was eliminated by the successive substitutions with less hydrophobic residues (Figure 2).

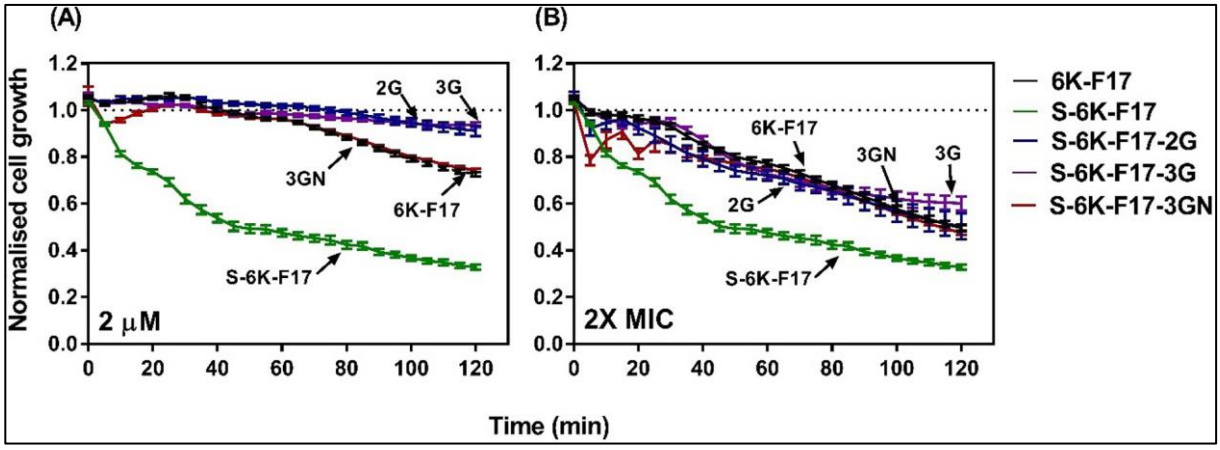

Figure 2. The effect of the peptides on time-dependent growth inhibition of E. coli *cells over two hours indicated that when cells were exposed to peptides at 2 μM (A) the stapled peptide S-6K-F17 had a higher inhibition rate whereas subsequent substitutions returned the rate to that of the parent (6K-F17). The stapled version S-6K-F17 was also more potent when peptides were used at double their individual minimum inhibitory concentrations (B). From Figure 4, Stone et al, 2018.*

NMR reveals further insights into peptide-membrane interactions

Studies using nuclear magnetic resonance (NMR) spectroscopy indicated that the helical 6K-F17 and non-helical S-6K-F17-3GN peptides bind to bacterial in similar ways and do not bind to mammalian membranes. The binding does not produce small micelle-like lipid particles, although binding may reduce lipid fluidity locally.

Elucidating the effect of stapling on the mechanism of action

The key observations were as follows:

- The hydrocarbon staple increases the rate and efficiency of membrane interaction *per se.*
- The staple drives the peptide further into the micelle interior, but this is reversed by substitutions with polar residues.
- Stepwise substitution with polar 'helix-breaking' residues draws the peptide out of both the micellar interior and the bilayer core.
- 6K-F17 and S-6K-F17-3GN specifically bind to negatively charged bacterial membranes.
- Incorporating two Gly residues decreases selectivity but including three Gly residues and an Asn improves selectivity, resulting in low toxicity up to as high as 450 μM.

A promising approach for peptide therapeutics targeting membranes

The results of this study clearly show that the critical balance between efficacy and toxicity of CAPs can be fine-tuned through the addition of a staple and also by substituting residues and rearranging sequence. Added to that, the method for cyclization could be easily adapted to most common helical peptides in an economical way on solid phase resin supports. The authors conclude that, “the ease of application and versatility of hydrocarbon stapling make the technique a promising avenue for the alteration and advancement of current peptide therapeutics, especially those designed to target and interact with biological membranes.”

Reference

1. The antibiotic resistance crisis: part 1: causes and threats. Ventola CL. Pharmacy & Therapeutics. 2015 Apr;40(4):277-83. https://www.ncbi.nlm.nih.gov/pubmed/25859123
2. Influence of hydrocarbon-stapling on membrane interactions of synthetic antimicrobial peptides. Stone TA *et al*. Bioorg Med Chem. 2018 Mar 15;26(6):1189-1196. doi: 10.1016/j.bmc.2017.10.020. Epub 2017 Oct 21. https://www.ncbi.nlm.nih.gov/pubmed/29275987