Peptide Stapling Applied to Antimicrobial Peptides

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Antimicrobial peptides (AMPs) are considered a promising therapeutic approach against multi-drug resistant microorganisms. Besides their advantages, there are limitations to be overcome so that these molecules can become market competitive. One of the biggest limitations is proteolytic susceptibility, which could be overcome by structural modifications such as cyclization, especially for helix-constraining strategies. Over the years, many helix stabilization techniques have arisen, such as lactam-bridging, triazole-based, N-alkylation and all-hydrocarbon stapling. All-hydrocarbon stapling takes advantage of modified amino acid residues and olefinic cross-linking to constrain peptide helices. Despite being a well-established strategy and presenting efficient stability results, there are different limitations especially related to toxicity. In the research, some studies on stapled AMPs for antimicrobial usage are explored with the aim of understanding the future of these molecules as putative antimicrobial agents.

antimicrobial peptides stapling all-hydrocarbon stapling

1. Introduction

Diseases and infections affecting humans, animals and plants are mostly managed by antimicrobial agents. Unfortunately, these antimicrobial agents, which are critical tools in our society, are becoming ineffective when facing resistant strains. The emergence of multi-drug resistant microorganisms has been a human and animal health threat since the discovery of resistant strains [1][2][3]. During the last three decades, antimicrobial peptides (AMPs) have demonstrated potential as antibiotic drugs able to face this global problem. Through wide-ranging research, natural and non-natural AMPs have been observed to kill bacteria through many mechanisms, including membranolytic and non-membranolytic modes of action, although the specific molecular targets for most peptides are still poorly described [4][5]. However, some physicochemical properties such as positive charge and amphiphilic properties have been widely associated with most peptides' antimicrobial activity [3].

Despite being interesting candidates, linear AMPs are usually unstable molecules that can rapidly undergo proteolytic degradation. Apart from their instability, they cause nonspecific membrane toxicity, presenting cytotoxic activity at low concentrations. These are by far the main limitations holding back the development of AMP-based antibiotic drugs [6][7][8]. Therefore, chemical modifications of AMPs are essentially oriented to improve their stability and antimicrobial activity and decrease cytotoxicity.

Several chemical modifications can be made to peptide structures to improve their properties, whether chemical, physical or biological. Nowadays, the main approaches proposed are insertion of D-amino acids, PEGylation, acetylation, dimerization, lipidation and cyclization [7][9][10][11][12][13][14][15]. Peptide cyclization is one of the most promising techniques to improve their stability and antimicrobial activity. Considering that AMPs' activity is mostly due to membrane disruption, restraining peptide conformation flexibility may facilitate insertion in the membrane by diminishing the entropic barrier related to the loss of freedom of molecular conformation [16][17]. Nevertheless, further in this review, flexibility aspects are discussed as being an interesting option for more efficient interactions.

Cyclization can be obtained via head-to-tail, side chain-to-head, side chain-to-tail or side chain-to-side chain bonding (**Figure 1**A) through different strategies [18][19]. Particularly for α-helical AMPs, but not exclusively [20], a cyclization approach called stapling has been used to constrain one face of the α-helix. Briefly, peptide stapling is characterized by cyclization between two side chain residues, resulting in a loss of peptide flexibility, which produces a more stable and less proteolysis-susceptible molecule (**Figure 1**B–E) [9][17][18].

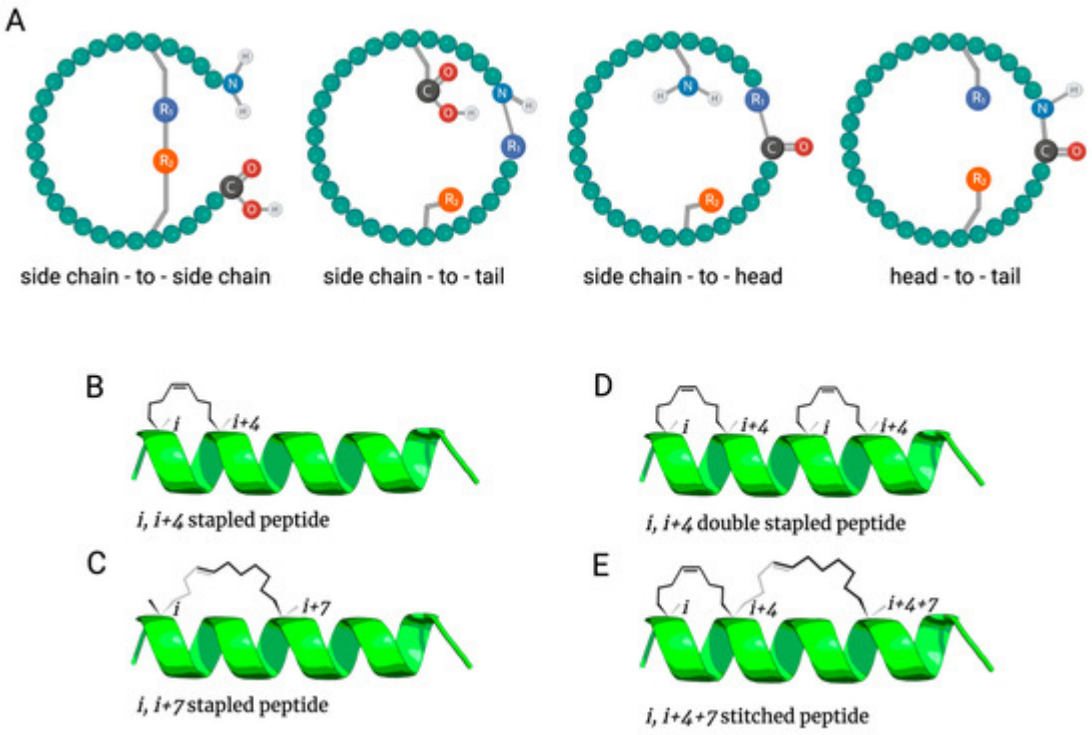

Figure 1. Cyclization and stapling strategies. Cyclization strategies (**A**) varying the linked positions in the peptide structure and stapling (**B**–**E**), which is a type of side chain-to-side chain cyclization, in which the most common positions are *i*, *i* + 4 (**B**) and *i*, *i* + 7 (**C**) for a single staple and possible positions for double stapling (**D**) and stitching (**E**).

Many stapling techniques attempt to constrain helical conformation in short peptides, tethering amino acid residues at *i*, *i* + 4 or *i, i* + 7 positions (where *i* is a given amino acid position). These stapling positions are more usual because naturally occurring proteins generally have their α-helices stabilized by intramolecular hydrogen bonds between the carbonyl and the amide groups of *i* and *i* + 4 residues, making the helix present around 3.6 residues

per turn. Therefore, such positions are able to extend the clamp to one (*i*, *i* + 4) (**Figure 1**B) or two turns (*i, i* + 7) of the α-helix (**Figure 1**C) in linear peptides [9][18][19][21][22]. Nonetheless, stapled helices at *i, i* + *11*, for three turns and *i, i* + *3* for a single turn are also viable in special cases [23][24].

Over the years, many helix stabilization strategies have arisen, from lactam bridging between the natural side chains of appropriately spaced amino acids to approaches using non-natural amino acids, including α, α-disubstituted amino acids, and many others like thiol-based, azide-alkyne cycloaddition, triazole and hydrocarbon stapling [18][25][26].

2. Antimicrobial Peptide Stapling

One focus of investigations into stapled peptides involves improving protein–protein interaction approaches for the development of anticancer and antiviral drug candidates [27][28][29][30][31][32][33][34][35]. Although stapling has not been widely used for AMP modifications, this technique has shown to be of great value in improving stability, and it is a promising technique for AMP rational design [36]. Despite a rising number of AMPs described, due to a series of drawbacks, only a few natural or non-natural AMPs available on different databases undergo clinical trials. The application of stapling strategies could change this scenario for the better [7].

The Data Repository of Antimicrobial Peptides (DRAMP) contains diverse annotated AMPs and an interesting classification method. Among all 22,259 entries, 5891 are general AMPs (natural and synthetic), 16,110 are from deposited patents, 77 are AMPs in drug development and 181 are stapled AMPs (**Figure 2**A). Analysis of the 181 stapled peptides revealed that the most common stapling positions are *i*, *i* + 4 or *i, i* + 7, accounting for 82.4% and 16.5%, respectively (**Figure 2**B). Another observation derived from this analysis is about the most popular stapling strategy, which is all-hydrocarbon stapling, accounting for 89.5% (**Figure 2**C) [37].

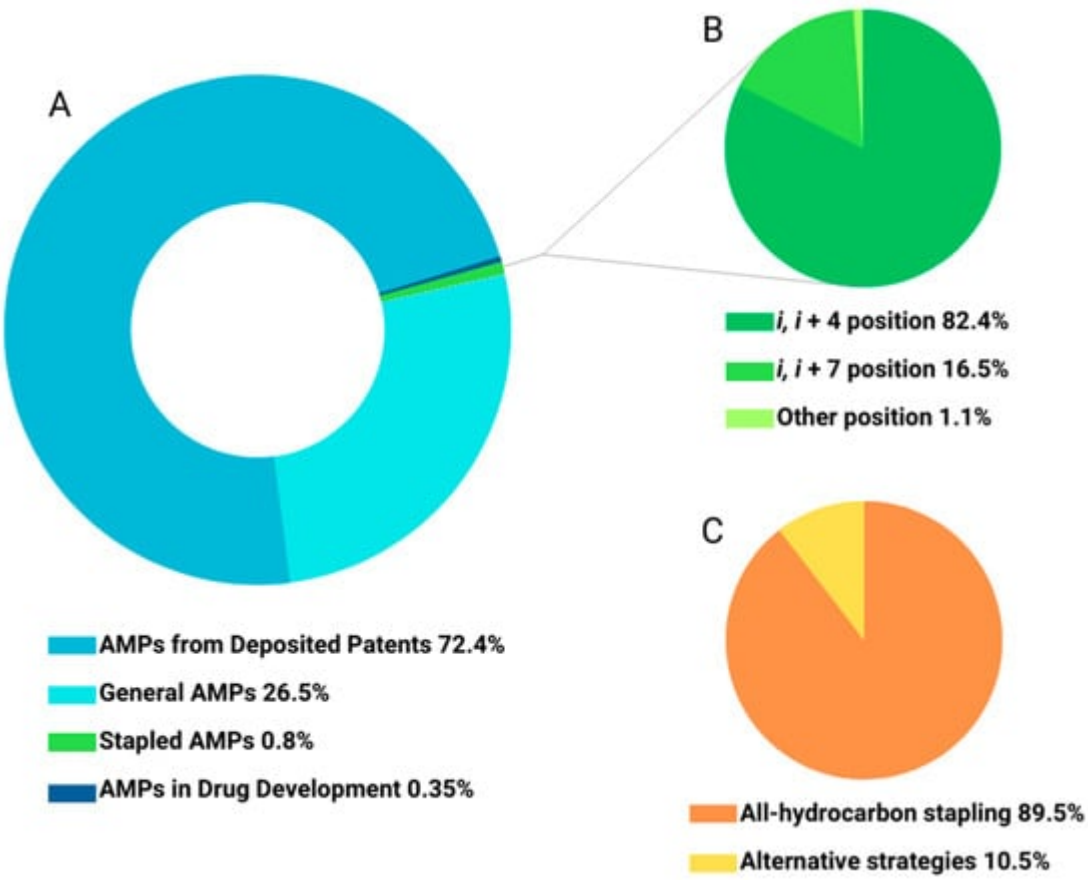

Figure 2. Classification of AMPs deposited on the Data Repository of Antimicrobial Peptides (DRAMP) describing (**A**) type of AMP, (**B**) most common positions for stapled AMPs and (**C**) stapling strategy applied.

2.1. All-Hydrocarbon Stapling

All-hydrocarbon stapling was first established by a research group led by Prof. Gregory Verdine [22]. Inspired by the development of the chemistry for olefinic cross-linking of helices, which is performed through *O*-allyl serine residues via ruthenium-catalyzed ring-closing metathesis (RCM), Verdine's group designed non-natural amino acids having either *R* or *S* stereochemistry at the α-carbon loaded with tethers of different lengths, followed by RCM and forming the desired clamp at determined peptide positions (**Figure 3**) [22][23][38]. The olefin tether bridge used for helix stabilization was considered similar to an actual staple, which explains why the term "staple" was introduced in this technique. Stapled helix stabilization relies on the tether length and absolute configuration at the α-carbon position. For this reason, these parameters have been intensely studied. Additionally, it is possible to constrain helices with more than one stapling, independent staples (**Figure 1**D) or via spiro-bicyclic ring connection, which is also called stitching (**Figure 1**E) [9][39][40][41].

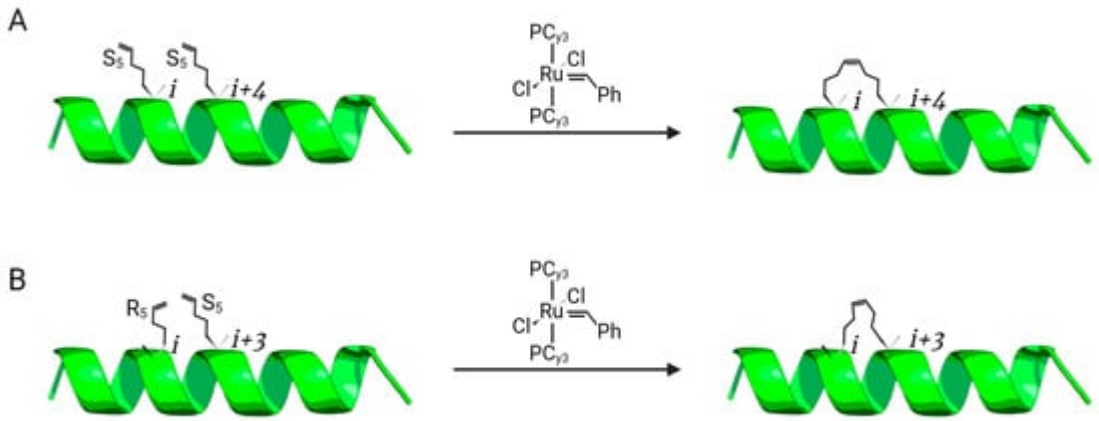

Figure 3. Cross-linking mediated by ruthenium-catalyzed ring-closing metathesis (RCM) between non-natural amino acids presenting S (**A**) and/or R (**B**) stereochemistry forming the staple at the desirable position within the α-helix structure.

Regardless of having both single and double stapling strategies available, single stapled peptides stand out in academic research [26][36][42][43]. The success of both strategies has shown to be peptide-dependent, as an increase in the helical content and improved antimicrobial activity is a premature correlation and not always observed to be true. Additionally, peptide stapling is often related to increased hemolytic activity, thus highlighting the importance of fine-tuning stapled AMP candidates.

The natural pro-inflammatory chemokine involved in response to microbial infections, CXCL10, is known to be active against different pathogens. However, there is evidence suggesting that CXCL10 have distinct antimicrobial domains. Taking this into account, Crawford et al. [38] mapped the chemokine structure and designed a series of nine overlapping peptides to test against a range of Gram-negative and positive bacteria. Among the derivative peptides, peptide P9, which was originated by the C-terminal α-helix of CXCL10, demonstrated by CD spectroscopy that it requires a specific conducive environment to adopt the native α-helix conformation. That way, in order to promote the secondary structure and enhance the associated antimicrobial activity, P9 was submitted to a stapling at positions 8 and 12, being substituted for the non-natural amino acid (S)-pentenyl alanine. As a result, a great improvement in bactericidal activity was observed, despite the resulting increase in cytotoxicity [44].

All-hydrocarbon stapled analogues of the naturally occurring peptide polybia-MPI (MPI) from the venom of the *Polybia paulista* wasp [45] were synthesized and had their chemical and biological properties evaluated by Luong et al. [43]. The three analogues (MPIS, MPIS-D8N, and MPIS-Q12K) have an oct-4-enyl hydrocarbon staple at the *i*, *i* + 4 positions, tethering amino acid residues 6 and 10, whereas MPIS presented only the stapling.

As a chemical modification, MPIS-D8N and MPIS-Q12K also had single amino acid substitutions. In MPIS-D8N, aspartic acid (D) in position 8 is substituted by asparagine (N), whereas glutamine (Q) 12 is substituted by lysine (K) in MPIS-Q12K. As a result, there was an increase of at least 3-fold in the α-helical contents of all analogues compared to their parent peptide, MPI. However, the antimicrobial activity was not necessarily better enhanced. All three analogues showed improved antibacterial activity between 7- and 23-fold against Gram-positive bacteria; still,

there was no improvement for the inhibition of Gram-negative bacteria. The hemolytic assay results revealed that all stapled peptides displayed an increase in hemolytic activity as follows: 12-fold for MPIS-D8N and approximately 4-fold for MPIS and MPIS-Q12K. In the last experiment, MPIS and MPIS-Q12K were submitted to a trypsin digestion assay and showed a 68-fold cleavage decrease [43].

As described above, hemolysis is an obstacle that must be overcome. For that reason, researchers seeking to develop and design stapled peptides should put effort into finding a well-balanced set of parameters, not only focusing on increasing hydrophobicity and helical content. In this line, Stone et al. [36] used the previously studied synthetic antimicrobial peptide 6K-F17 (KKKKKK-AAFAAWAAFAA-NH_2) [46][47] to generate four stapled analogues; all of them clamped at the same position, but three of them did so with amino acid substitutions. The stapled S-6K-F17 with no amino acid substitution showed a higher α-helix formation tendency, increased antimicrobial activity against *E. coli* BL21, and a more rapid killing mechanism compared to the non-stapled version (6K-F17) and the other three stapled analogues with amino acid substitutions (S-6K-F17-2G, S-6K-F17-3G and S-6K-F17-3GN). Nevertheless, the observed hemolytic activity of S-6K-F17 was much higher, with an increase varying between 4- and 154-fold. However, the three stapled analogues with higher-polarity amino acid substitutions, namely glycine and/or asparagine, despite not presenting so much helicity, were less toxic to mammalian cells and had only minor antimicrobial activity shifts, 0- to 3-fold. That observed outcome is probably due to a better hydrophobicity balance and selectivity for prokaryotic membranes, since adding staples increases non-specific membrane interactions. These results highlight those constraining helixes, and although they have a high tendency to improve antimicrobial activity, they should not be the only focus in staple studies. Adding polar amino acids, which are unfavorable for helix formation and decrease the helicity index, helps with selectivity and thus with toxicity aspects.

Proteolytic stability is a crucial characteristic for AMP development. Although stapled peptides usually present higher resistance to enzymatic degradation, this property is also dependent on the composition of peptide amino acid residues. Aiming to understand the influence of stability, helicity and antimicrobial activity against pathogenic fungi using an all-hydrocarbon strategy, Zheng et al. [42] developed single-stapled analogues at *i*, *i* + 4 positions using aurein 1.2, a peptide secreted by the Australian bell frog *Litoria aurea* [48]. After CD spectroscopy analyses, most stapled peptides showed an increase in their helical content. However, this increase did not necessarily lead to an improvement in antifungal activity against *Candida* spp. strains. Four stapled analogues showed better antifungal activity and were selected to undergo a protease stability test by chymotrypsin degradation. Data indicate better proteolytic protection within all selected analogues compared to aurein 1.2. However, among these analogues, one presenting a higher helicity index showed lower proteolytic stability. Therefore, the results indicate that, despite achieving a boosted aurein 1.2 analogue, improving a peptide's antimicrobial potency and stability can be more complex than just constraining helices [42].

Besides all the difficulties that could be found, stapling continues to be a promising strategy for AMP rational design. Hirano et al. [26] decided to improve the potential of a peptide 1 drug, a previously reported Magainin 2 (Mag 2) derivative, by designing and synthetizing 8 side chain stapled peptides, numbered from 2 to 9, in order to evaluate their preferred secondary structures, and their antimicrobial and hemolytic activities. These peptides were synthesized by introducing a suitable (S)-pentenyl alanine and/or (R)-octenyl alanine at the *i*, *i* + 4 or *i*, *i* + 7

positions, which would lead to side chain stapling. As a result, peptides 2 to 6, presenting *i*, *i* + 4 stapling, showed improved antimicrobial activity, while peptides 7 to 9, presenting *i, i* + 7 stapling, had decreased or indifferent shifts in antimicrobial activity. The investigation of hemolytic activity showed that among all the stapled peptides, only peptide 2 demonstrated hemolytic activity at much higher concentrations compared to MIC, estimated as being 16× higher for gram-positive *S. aureus* and 32× higher for gram-negative *E. coli* and *P. aeruginosa*. All the other peptides presented hemolytic activity at concentrations as low as MIC or even lower, and peptide 8 was the one with the lowest hemolytic concentration. In addition, electrophysiological measurements were performed, revealing that peptide 2 showed higher scores against the DOPE/DOPG membrane than against the DOPC membrane. These results indicate a pore-formation preference for bacterial mimetic than for mammalian mimetic membranes.

Understanding the possible drawbacks related to characterizing stapled peptides, the importance of studies that pursue more complex and rich evaluations is clear, especially those taking advantage of computational approaches. Thus, it can be possible to generate better knowledge and insights on how to improve stapled peptides' therapeutics and competitiveness.

2.2. Alternative Stapling Strategies

Although it is the most used stapling technique, hydrocarbon stapling is only one of the many approaches available. The term "stapling" has become a general term to refer to cyclic peptides, especially those that aim to constrain helices [19]. Alternatively, there are one-component approaches such as disulfide or amide bonding, and two-component methodologies, including thioether formation and alkylation of Lys residues [17]. In this perspective, Liu et al. [49], also using polybia-MPI as the original counterpart, generated the stapled analogues C-MPI-1 and C-MPI-2 by a triazole stapling technique. From these, only C-MPI-1 presented a slight α-helical structure. Even though C-MPI-1 showed higher helicity content, there was little or no enhancement in antimicrobial activity against Gram-positive and Gram-negative bacteria and worse hemolysis results compared to MPI.

Similarly, the cationic AMP OH-CM6, derived from the natural cathelicidin peptide OH-CATH30 from the king cobra *Ophiophagus hannah* was used to generate stapled analogues by N-alkylation of lysine [50]. In this strategy, the lysine residues on the hydrophilic face of the native peptide were clamped by an alkyl linker at *i*, *i* + 4 and *i, i* + 7. During this process, ten stapled peptides were generated and screened for antimicrobial activity against Gram-positive and Gram-negative bacteria, including clinical isolated strains. The best candidate (peptide 10) was chosen after antimicrobial screening. From this selected peptide, different analogues were synthesized with an aralkyl linker. One of these peptides (peptide 12) presented antibacterial activity against methicillin-resistant *S. aureus* in concentrations comparable to Vancomycin. In addition to that, despite the toxicity observed in the assay against the HEK 293T cell line, the concentration was 32-fold higher than the observed minimum inhibitory concentration, showing an interesting therapeutic index. Moreover, the α-helical content was around 45% and the proteolytic stability was better than other analogues tested, namely the linear and one of the stapled analogues, indicating a promising molecule for further investigation.

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