Bioorganic & Medicinal Chemistry 26 (2018) 1203–1205

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

A study of 2-component i, i + 3 peptide stapling using thioethers

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ARTICLE INFO

Article history:
Received 15 September 2017
Revised 20 October 2017
Accepted 28 October 2017
Available online 31 October 2017

ABSTRACT

Peptides are promising scaffolds for use as therapeutics, targeting interactions previously considered to be "undruggable" by small molecules. While short peptides are generally unstructured in solution and rapidly degraded by proteases in the cell cytosol, peptide stapling offers an effective method to both stabilize peptides in a helical structure and increase resistance to proteolytic degradation. Most studies of peptide stapling have focused on residues with i, i + 4 and i, i + 7 spacing, while stapling of residues with i, i + 3 spacing has been understudied. Herein, we evaluated a suite of bifunctional linkers for stapling between residues with i, i + 3 spacing, comparing the ability of each compound to react with the peptide and the degree of helicity conferred. Finally, we evaluated the ability of the stapling to increase proteolytic resistance in cell lysates, comparing stapling of i, i + 3 and i, i + 4 spacing, with i, i + 3 spacing resulting in a greater increase in peptide half-life in the model system. This presents an effective stapling strategy, adding to the peptide stapling toolbox.

1. Introduction

1.1. Peptides as therapeutics

Peptides possess many attractive qualities for use as therapeutics. The extended nature of peptides compared to traditional small molecule drugs increases target selectivity, and the native peptide backbone increases both biological compatibility and clearance from the body.[1,2] In particular, the larger surface area of peptides allows access to "undruggable" targets: disruption of protein-protein interactions (PPI).[2] The extended surfaces at the interface, as well as the conformational requirements for high affinity binding in many PPIs, makes targeting of these interactions by small molecules largely intractable.

However, like all tools, peptides have limitations. These short amino acid sequences are generally unstructured in solution, which can result in lower affinity binding to the target protein. Additionally, peptides are highly susceptible to proteolytic degradation. There are many methods for overcoming these pitfalls, such as the addition of unnatural amino acids,[3] cyclization of the peptide,[4] and the introduction of a covalent bond between two amino acids, commonly referred to as peptide stapling.[5]

1.2. Peptide stapling

Peptide stapling both stabilizes the peptide in an α-helical conformation and protects the peptide from proteolytic degradation. Several different chemistries have been applied to peptide stapling, such as ring-closing metathesis,[6] lactam formation,[7] copper-assisted azide-alkyne cycloaddition,[8,9] and thioether formation.[10–16] Many of these chemistries can be applied either to the direct cyclization of two amino acids or mediated through a bifunctional linking agent, also referred to as two component stapling.[5] Most applications of peptide stapling have focused on linking amino acids with i, i + 4 or i, i + 7 spacing.[5] When mutating amino acids to include the appropriate functionality for the stapling reactions, there are times that stapling amino acids with i, i + 3 spacing would be ideal. While i, i + 3 spacing has been applied to ring-closing metathesis,[17] disulfide formation,[18] and direct thioether formation between two unnatural amino acids,[10] and was also identified in a diversity-oriented screen of stapling postions,[19] this stapling position is understudied. Here, we use bisalkylation of two cysteine residues and scan a suite of alkyl halides for the ability to stabilize helical structure in a short model peptide with i, i + 3 spacing. Additionally, stapling with i, i + 3 or i, i + 4 spacing is compared in the context of proteolytic resistance in cell lysate.

2. Results and discussion

2.1. Stapling efficiency

Peptide stapling in the form of cysteine alkylation is attractive for a number of reasons. The use of natural cysteine residues allows the stapling chemistry to be applied to biologically expressed peptides[20] or those synthesized using solid phase peptide synthesis. As this is mediated through a bifunctional linking

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https://doi.org/10.1016/j.bmc.2017.10.037
0968-0896/

agent, it also presents the possibility for further manipulation of the linker,[21] such as attachment of a fluorophore or cell-penetrating peptide.

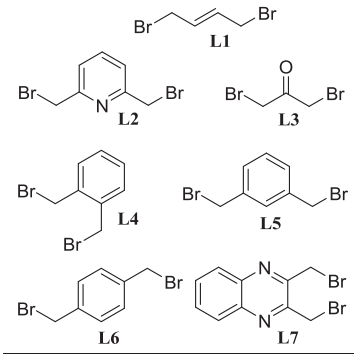
A model peptide sequence that had been previously used to study Cys stapling with i, i + 4 spacing[11] (Table 1) was modified to incorporate i, i + 3 spacing between the cysteines. A suite of alkyl halides was selected that had previously been investigated for i, i + 4 stapling (Fig. 1). While L5 had been reported to be optimal for i, i + 4 spacing, given the shorter distance between i, i + 3 residues relative to i, i + 4 spacing (Cα-Cα distance of 5.1 Å and 5.9 Å, respectively), we questioned whether a different linker may be preferred. Compounds L1–L7 were reacted with Ac-P1 to determine which would result in the cleanest conversion to stapled peptide (Fig. 1). Dibromobutene (L1) resulted in the highest conversion to stapled peptide (93% based on HPLC peak integration) and no by-products. Both bis(bromomethyl)pyridine (L2) and dibromoacetone (L3) resulted in similar conversion, 95% and 94%, respectively, but the mass spectrum contained masses of unidentified by-products. All of the bis(bromomethyl)benzene (L4–6) compounds reacted cleanly to varying degrees of conversion, with the *meta* substitution (L5) resulting in the best conversion (89%). Bis(bromomethyl)quinoxaline (L7) gave the lowest yield of stapled peptide, at 52%, and resulted in some monohydrolysis of the compound, suggesting hydrolysis of the bromomethyl group was competitive with alkylation of cysteine.

2.2. Helical propensity

In addition to the reactivity of the various compounds, the degree of α-helicity conferred by each compound was measured. Circular dichroism was used to determine the secondary structure of the peptides, and percent helicity was calculated based on the mean residue ellipticity at 222 nm. As seen in Fig. 2, Ac-P1, Ac-P1·L4, Ac-P1·L7, Ac-P1·L3 are random coil, indicated by a single minimum at 195 nm. The remaining compounds confer some degree of α-helical character with two minima at 222 and 208 nm, with L6 conferring the greatest degree of helicity at 40% (Fig. 1).

Table 1
Peptide sequences.

Peptide	Sequence
Ac-P1	Ac-YGGEAAREACARCEAARE-NH2
FAM-P1	FAM-YGGEAAREACARCEAARE-NH2
FAM-P2	FAM-YGGEAAREACARECAARE-NH2



Compound	% Conversion	% Helicity
L1	93	28
L2	95	8
L3	94	8
L4	82	3
L5	89	26
L6	65	40
L7	52	5

Fig. 1. Suite of linkers for 2-component stapling and table of stapling conversion and helicity. Peptides (1 mM) were stapled in 100 mM ammonium bicarbonate buffer, pH 8 with 1.5 eq. TCEP and 3 eq. linking agent (in DMF) for 2 h at room temp. CD conditions: 50 μM peptide in 10 mM Na phosphate, pH 6.5.

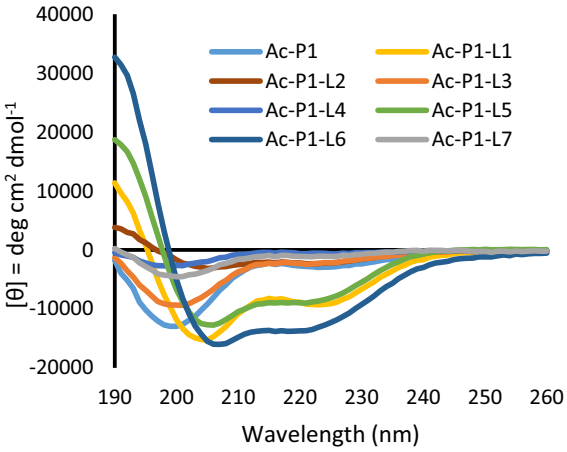


Fig. 2. Circular dichroism spectra of unstapled peptide Ac-P1 as well as stapled peptides Ac-P1·L1 – Ac-P1·L7. 50 μM peptide in 10 mM Na phosphate, pH 6.5.

2.3. Proteolytic stability

Peptide stapling is used to not only stabilize secondary structure, but it also helps to protect peptides from proteolytic degradation. Proteases and peptidases in the cytosol of cells rapidly degrade short, unstructured peptides, so we compared the effectiveness of stapling with i, i + 3 spacing to that of i, i + 4 spacing for conferring proteolytic stability relative to their unstapled counterparts. Moving forward with the model peptide, the 1,3-bis(bromomethyl)benzene linker (L5) was used for stapling as it resulted in one of the best combinations of conversion to stapled product and helical content, and similar results have been shown with this compound in the context of i, i + 4 stapling.[11]

Proteolytic stability was determined by incubating both stapled and unstapled peptides in cell lysate, a more harsh environment than the cytosol of cells or serum. Upon analysis using analytical RP-HPLC with fluorescence detection, the percent of intact peptide was measured at each time point, allowing for determination of the peptide half-life. The stapled and unstapled versions of FAM-P1 were run in parallel, showing an ~3-fold increase in peptide half-life (7 min vs 21 min, respectively). This demonstrates stapling of residues with i, i + 3 spacing is a viable option for conferring resistance to proteolytic degradation. Surprisingly, stapling does not appear to affect the proteolytic stability of FAM-P2 in the context of this peptide sequence, as FAM-P2 has a half-life of 16 min, while FAM-P2·L5 has a half-life of 10 min (Fig. 3). We originally hypothesized that if any difference in the increase of protease resistance upon stapling were seen between the stapling positions, it would be in favour of the i, i + 4 spacing, as the linking compound covers one additional amino acid; however, we observed the opposite. Some proteases have sequence specific cleavage sites, and the slight difference in the sequence of FAM-P1 and FAM-P2 could explain the changes in the fold increase between stapling positions. As peptide sequence plays a role in proteolytic susceptibility, this work demonstrates the important of investigating different stapling positions in the system of interest.

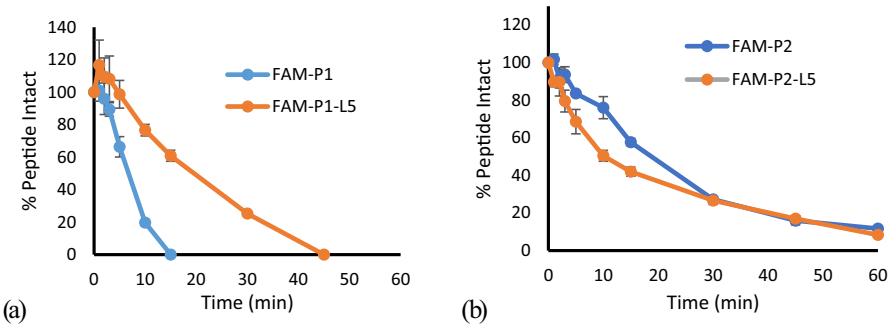


Fig. 3. Cell lysate degradation. Conditions: 10 μM peptide, 3 mg/mL total [protein], 37 °C.

3. Conclusion

Here we have scanned a suite of bifunctional linkers for the stapling of cysteine residues with i, i + 3 spacing, finding that several compounds led to high conversion to stapled peptide, but only three compounds resulted in peptides with increased α-helical character. The proteolytic resistance of one of the stapled peptides was determined in cell lysates, showing that i, i + 3 stapling does not result in any loss of resistance compared to i, i + 4 spacing; rather, it conferred a greater degree of resistance in this model peptide when compared to its unstapled counterpart. This study presents a viable option for peptide stapling when mutation of residues for stapling causes less disruption of the sequence when the residues are spaced by three amino acids, further diversifying the peptide stapling toolbox and aiding the development of peptide-based therapeutics.

4. Experimental methods

4.1. Peptide synthesis, stapling, and purification

Peptides were synthesized on a 0.1 mmol scale by Fmoc solid phase peptide synthesis using Rink amide resin. Briefly, Fmoc deprotections were accomplished with 20% piperidine. Amino acids were coupled twice using HBTU/HOBt and DIPEA. After the final Fmoc removal, N-termini were either acetylated (6% 2,6-lutidine, 5% acetic anhydride 30 min) or capped with carboxyfluorescein (4 eq. FAM, 5 eq. PyBOP/HOBt, 8 eq. DIPEA 4 h + overnight). Final deprotection and cleavage was accomplished with 2.5% TIPS, 2.5% EDT, 1% water, 94% TFA. Peptides were purified using semi-preparative RP-HPLC.

For stapling, peptides were dissolved in 100 mM ammonium bicarbonate buffer, pH 8 to a final concentration of 1 mM. 1.5 eq. of TCEP were added and stirred 1 h, followed by 3 eq. of each linker dissolved in DMF. After 2 h the reaction was quenched with addition of 0.5 M TFA, and stapled peptides were purified using RP-HPLC. The percent conversion was determined by integrating the peak area of stapled and unstapled peptide.

4.2. Circular dichroism

Circular dichroism was collected on a Chirascan Plus. Peptides were dissolved in 10 mM sodium phosphate buffer, pH 6.5 to a concentration of 50 μM. Mean residue ellipticity was calculated using the equation $MRE = \frac{\theta}{10lc}\left(\frac{1}{n}\right)$, where θ is the ellipticity, l is the path length, c is the concentration, and n is the number of residues. The percent helicity was calculated using the equation $\%H = \frac{\theta_{222\,obs} - \theta_{222\,0\%}}{\theta_{222\,100\%} - \theta_{222\,0\%}}$, where $\theta_{222\ obs}$ is the observed MRE at 222 nm, $\theta_{222\ 0\%}$ = 640 deg cm^2/dmol, and $\theta_{222\ 100\%}$ = −34,444.44 deg cm^2/dmol.

4.3. Cell lysate degradation

293T cell pellets were resuspended in PBS and lysed using a homogenizer. Total protein concentration was determined using Bradford assay. Assays were run in triplicate with a lysate final protein concentration of 3 mg/mL. Peptides were dissolved in PBS, as was free carboxyfluorescein (FAM) to be used as an internal standard. Final peptide and FAM concentrations were both 10 μM. The assay was initiated upon addition of the peptide and incubated at 37 °C. Aliquots were taken at the indicated times and quenched with an equivalent volume of 200 mM HCl. The zero time point was prepared by first quenching lysate with 200 mM HCl, followed by addition of the FAM standard and peptide. Assays were analysed by analytical RP-HPLC-FD. The unstapled peptide formed some peptide dimer linked by a disulfide. The calculated intact peptide included both intact monomer and intact disulfide linked dimer.

Acknowledgements

We gratefully acknowledge the UNC Lineberger Comprehensive Cancer Center for funding of this work. We also thank the Allbritton lab at UNC for the generous use of their HPLC with fluorescence detection, and the Williams lab for the 293 T cells.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.10.037.

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