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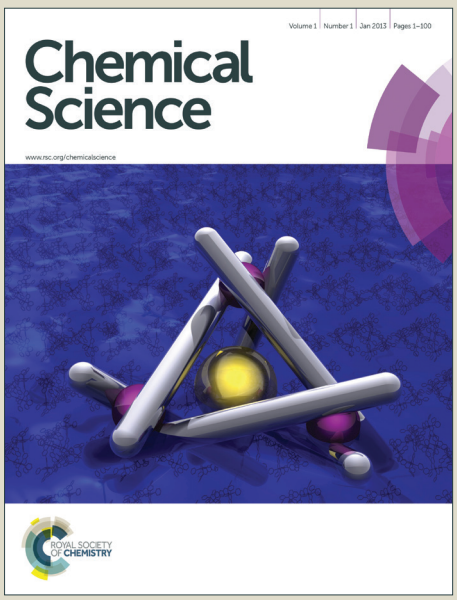

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EDGE ARTICLE

Functionalised staple linkages for modulating the cellular activity of stapled peptides

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Stapled peptides are a promising class of alpha-helix mimetic inhibitors for protein-protein interactions. We report the divergent synthesis of 'functionalised' stapled peptides *via* an efficient two-component strategy. Starting from a single unprotected diazido peptide, dialkynyl staple linkers bearing different unprotected functional motifs are introduced to create different alpha-helical peptides in one step, functionalised on the staple linkage itself. Applying this concept to the p53/MDM2 interaction, we improve the cell permeability and p53 activating capability of an otherwise impermeable p53 stapled peptide by introducing cationic groups on the staple linkage, rather than modifying the peptide sequence.

Introduction

Many cellular functions are governed by complex networks of protein-protein interactions (PPIs). Compounds which are able to inhibit specific PPIs are vital tools in chemical biology for elucidating the role of individual proteins in a large network. Furthermore, developing general methods of inhibiting PPIs may open up whole new classes of therapeutic protein targets, going beyond the traditional 'druggable' genome of predominantly enzymes and receptors.[1]

One major challenge for developing inhibitors of PPIs is the lack of natural small molecule binding partners from which inhibitors can be designed.[2] At the same time, high throughput screens often fail to provide hits, as the typical 'rule of five' compliant compounds found in many chemical libraries are often poor candidates for binding protein-protein interfaces.[3] An alternative approach is the synthesis of secondary structure mimetics, using the native protein sequence as a template for designing new inhibitors. There are a number of effective peptidomimetic strategies reported in the literature.[4] In particular, Grubbs, Verdine, Walensky and Sawyer have established a promising class of mimetics known as stapled peptides,[5] in which two non-proteogenic amino acids bearing alkenyl side chains are joined by ring-closing metathesis to constrain a peptide into an alpha-helical conformation. Stapling peptides has been shown to improve binding affinity and pharmacokinetic properties when compared to the native peptide sequence for several different PPI targets in which the interface involves a helical motif.[5]

Despite the successes of this methodology, there is no guarantee that stapling will endow a peptide with improved properties. In some cases, stapled peptides will have a lower affinity for their protein target,[6] or be unable to enter cells.[6b,7] Given these caveats, many literature studies on stapled peptides begin with optimisation of linker length and position to find the best staple orientation.[5,8] After achieving a high affinity binder *in vitro*, further alterations in the peptide sequence itself are often carried out to achieve cell-permeability and cellular activity, whilst taking care not to compromise affinity and specificity.[7,9]

For macrocyclisation stapling techniques such as hydrocarbon stapling,[10] each variation in the staple length, staple position or peptide sequence requires a new linear peptide to be synthesised, as all these parameters are predetermined by the choice of non-natural amino acids during solid phase peptide synthesis (Figure 1A). We reasoned that a more efficient stapling method would involve two components, the peptide and a separate stapling linker, which combine to form the final stapled peptide (Figure 1B). In this case, it is possible to start from a single linear peptide and generate a collection of stapled peptides with different properties based on the nature of the linker. At present, Lin,[9] Greenbaum[11a] and Inouye[11b] have used two-component approaches to screen structurally different linkers, finding the optimal linker length for maximal helicity or stapling reactivity. In the context of general peptide macrocycles (not necessarily alpha helices), Timmerman, Pentelute, Horne, Fasan and Suga have explored variable-length linkers for generating different cyclic

scaffolds.[12] We now demonstrate that introducing different functionality on the staple linker can improve the cellular uptake and activity of stapled peptides, without needing to alter the peptide sequence.

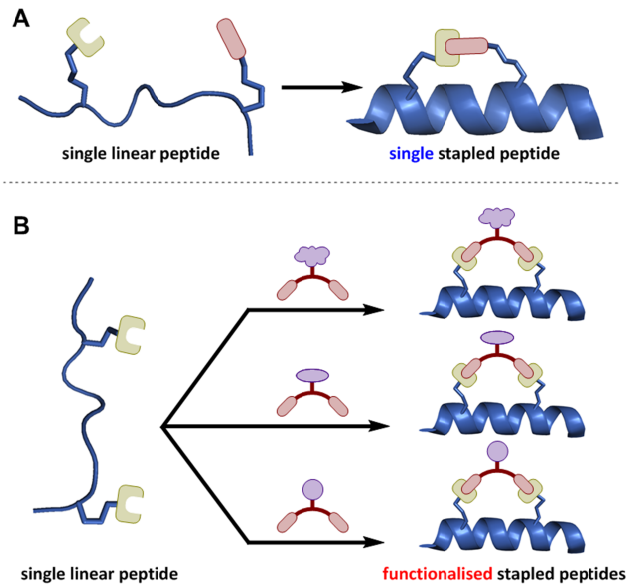

Figure 1. A) Most stapled peptides are formed by macrocyclisation between two functional groups on a peptide. B) A two component stapling strategy in which the peptide has two sites of reactivity (blue), and external linkers have two corresponding sites (red). Different stapled peptides can be divergently generated using linkers with different functionality (oval, star and rectangle).

We envisaged the linker as a handle for further reactivity, onto which a variety of functional motifs could be appended. The existing two-component reactions used by Lin,[9] Greenbaum[11a] and Inouye[11b], as well as the CLIPS technology developed by Timermann[12a] and the polyfluorobenzene linkers by Pentelute,[12b] involve cysteine or lysine alkylation/acylation chemistry. Whilst the proteogenic amino acids cysteine and lysine are an advantage in terms of synthesis or genetic encoding, and in some cases the catalyst-free nature of cyclisation, cross-reactivity may arise in the presence of other nucleophilic functional groups, or from sulphur oxidation. Hence, we sought to develop a more chemospecific and functional group tolerant reaction for the stapling process. This eliminates any unwanted side reactions arising from functional groups appended to the linkers, whilst avoiding the need for extra protecting groups. We therefore chose to introduce azides into our peptides for their bioorthogonality (Figure 2A),[13] as well as their ease of synthesis from naturally occurring amino acids.[14] Corresponding dialkynyl linkers **1-5** (Figure 2B) for Cu-catalysed azide-alkyne cycloaddition[15] were designed to be symmetrical and achiral to avoid the formation of regioisomers and diastereomers upon peptide stapling.

Results and Discussion

Development of two-component stapling methodology

To test that our stapling method was capable of inducing helicity in non-helical linear peptides, we first used a model i, i+7 diazido peptide **MP0** similar to one used by Inouye and coworkers.[11b] We chose to conduct the stapling in solution phase to avoid issues of site isolation, as our preliminary studies with on-resin two-component stapling showed poor conversion with increasing sequence length. Our initial stapling conditions involved subjecting the unprotected peptide to an excess of dialkynyl linker, copper(II) sulphate and sodium ascorbate in a 1:1 mixture of acetonitrile and 20 mM sodium phosphate buffer at pH 7.6. We found that stapling with 3,5-dialkynylbenzene linker **1** increased the helicity of the peptide from 16% to 51%, as estimated by circular dichroism at 222 nm (Figure 3).

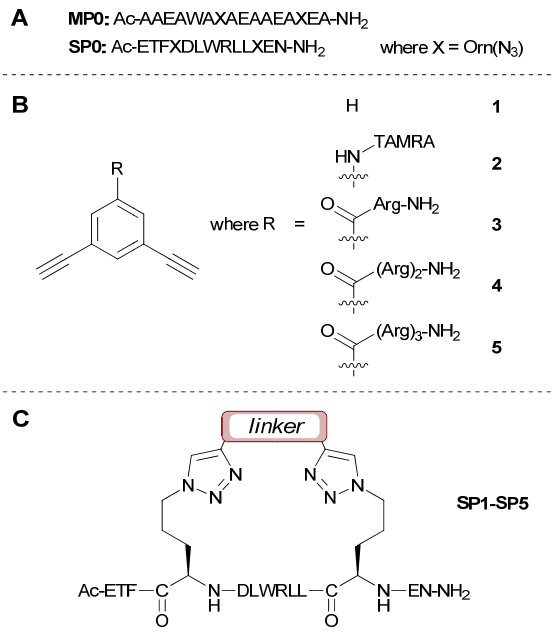

Figure 2. A) Diazido peptides **MP0** (model i, i+7 peptide) and **SP0** (based on residues 17-29 of the p53 sequence). B) Functionalised dialkynyl linkers **1-5**. C) General structure of the bis-triazolyl stapled peptide product. TAMRA = 5-carboxytetramethylrhodamine, Arg = arginine. All other amino acids are represented using their standard single letter code.

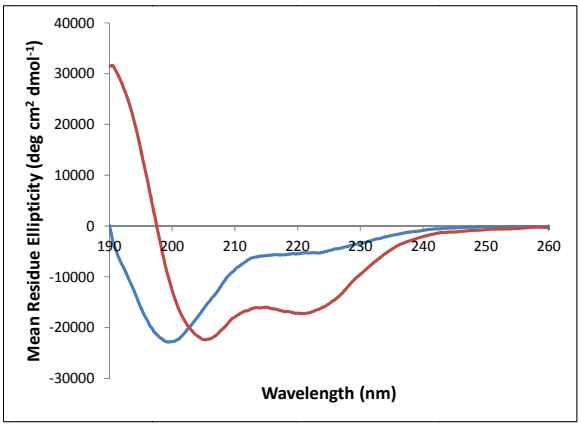

Figure 3. Circular dichroism spectra of linear model peptide **MP0** (in blue) and stapled model peptide **MP1** (in red).

With this initial success in our model system, we then decided to target the p53/MDM2 interaction, a promising therapeutic target for cancer therapy.[16] Inhibitors which block this PPI can prevent ubiquitination of p53 by MDM2, and free the transactivation domain of p53, thereby restoring p53 function in p53 wild-type cancer cells.[16c] Whilst we[6b] and others[5e] have previously had success developing stapled peptide inhibitors of this PPI using peptide sequences derived from phage display, we specifically chose to apply our new stapling method on a p53 peptide derived by substitution of the wild-type sequence, previously reported to be cell-impermeable even after hydrocarbon stapling.[7a] For such peptide sequences, cell-permeability can be achieved by mutating away anionic amino acids and introducing cationic residues.[7,9,17] However, we aimed to functionalise the staple as an alternative way of optimising cell permeability, independent of changing the peptide sequence.

Starting from a single fully unprotected p53-based peptide **SP0**, we attempted the stapling with linker **1** using our initial stapling conditions. However, a large amount of starting material remained unreacted after several days, despite the addition of extra reagents. Changing the stapling conditions to one equivalent of linker, copper(II) sulphate, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) as a ligand and three equivalents of sodium ascorbate in 1:1 water/tert-butanol gave complete conversion to the stapled peptide. Using these improved stapling conditions, five different stapled peptides **SP1**-**SP5** were synthesised in one step by introducing the linkers **1**-**5**. Importantly, the stapling reaction proceeded cleanly in all cases with exceptional functional group specificity and tolerance. (Figure 4) Furthermore, no oligomerised and non-cyclised linear coupling products were observed (Supporting Information 4.3).[18]

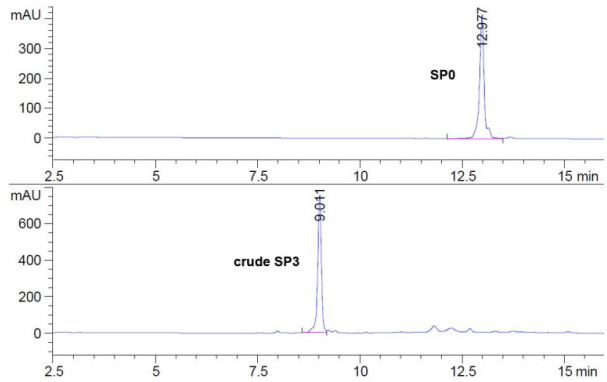

Figure 4. HPLC chromatographs of pure starting peptide **SP0** (top), and the crude reaction mixture after stapling with linker **3** to give stapled peptide **SP3** (bottom), monitored at 220 nm.

Biophysical comparison of stapled and unstapled peptides

Stapled peptide **SP1** showed high affinity for binding MDM2, as determined by competitive fluorescence polarisation and isothermal calorimetry (Table 1). Whilst the binding affinity is greatly improved over the wild type $p53_{17-29}$ peptide, the improvement over **SP0** is more modest. We also note that the binding affinity of **SP1** compares favourably to that previously reported[6b] for the related hydrocarbon stapled peptide SAH-8 (50.2±5.5 nM), which has several rationally-designed mutations from the wild-type sequence. The high affinity of **SP0** itself originates from the replacement of a Pro-27 residue in the wild type peptide with the azido amino acid in both **SP0** and **SP1**. Proline has a poor helix-propensity, and molecular dynamics simulations indicate that the helix does not extend past Leu-25 for the wild-type peptide, whilst the helicity extends through to Glu-28 for both **SP0** and **SP1** (Supporting Information 7). These results are also consistent with previous mutational studies on p53 peptides conducted by Zondlo and coworkers, where replacing Pro-27 with a serine significantly improved binding affinity towards MDM2.[19]

Table 1. IC_{50} values and binding affinities for peptides determined by competitive fluorescence polarisation (FP) and isothermal calorimetry (ITC).

Peptide[a]	FP IC_{50} (nM)	FP K_i (nM)	ITC K_d (nM)
wt $p53_{17-29}$	4807±594	821±56	483±79
SP0	161±7.7	16.1±1.2	44.3±9.0
SP1	88.5±3.0	3.21±0.38	6.7±2.8
SP3	90.2±3.4	3.73±0.42	7.3±1.8
SP4	121±4.5	7.97±0.69	9.6±2.5
SP5	149±5.4	11.7±0.91	29.8±5.2
RRR-SP0	268±12	32.5±2.1	15.2±5.0[b]

[a] Peptide **SP2** was not compatible with the competitive assay due to its TAMRA-labelled linker, so the K_d was determined by direct fluorescence polarisation to be 28.0±7.2.

[b] When the ITC data for **RRR-SP0** was fitted to a single-site model, the resulting curve fitting gave an N value of 1.97, suggesting there may be other interactions involved.

In addition, the wild type peptide displays a random coil signal by circular dichroism, whilst both **SP0** and **SP1** display alpha-helical circular dichroism spectra (Supporting Information 4.5), with **SP0** in fact showing greater helicity. However, one property which is enhanced by the stapling process is the proteolytic stability of the peptide. **SP0** was found to have poor *ex vivo* serum stability, with only 18% intact peptide remaining after a 30 h incubation period (Figure 5). In contrast, **SP1** showed excellent stability with 79% intact peptide under the same conditions, highlighting the importance of the stapling process.

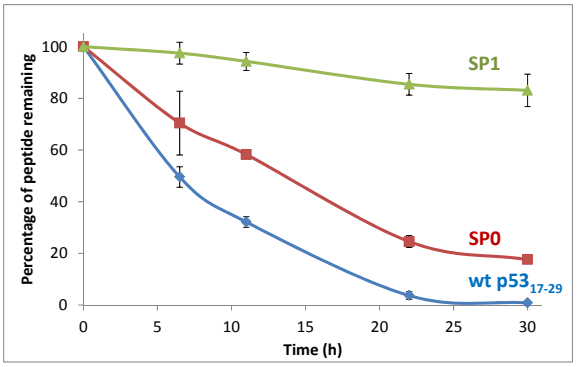

Figure 5. Serum stability peptides incubated in mouse serum at 37 °C.

Cellular activity of p53 peptides

To investigate the efficacy of our stapled peptides to activate p53 in a cellular environment, we decided to evaluate the cell permeability of our stapled peptides. **SP2** was designed as a fluorescently-labelled version of **SP1**, demonstrating the ability to simultaneously staple and label a peptide in one step using our two-component methodology. When human osteosarcoma U2OS cells were incubated with 50 μM **SP2** for 24 h, no fluorescence was observed in cells by confocal microscopy, consistent with the previously reported hydrocarbon stapled p53 peptides based on the same amino acid sequence.[7a] Utilising the staple linkage as a handle for chemical functionalisation, we then incorporated a variable number of arginine residues on linkers **3-5** as cell-permeabilising motifs. Cationic stapled peptides **SP3-SP5** exhibited comparable helicities and binding affinities to MDM2 (Table 1 and Supporting Information 4.5), confirming that the linker modifications could be made without significantly disturbing the biophysical properties of the stapled peptide. Confocal microscopy on *N*-terminal dye-labelled versions of **SP3-SP5** indicated cellular uptake of the peptides when up to three arginines were introduced on the linker component (Figure 6).

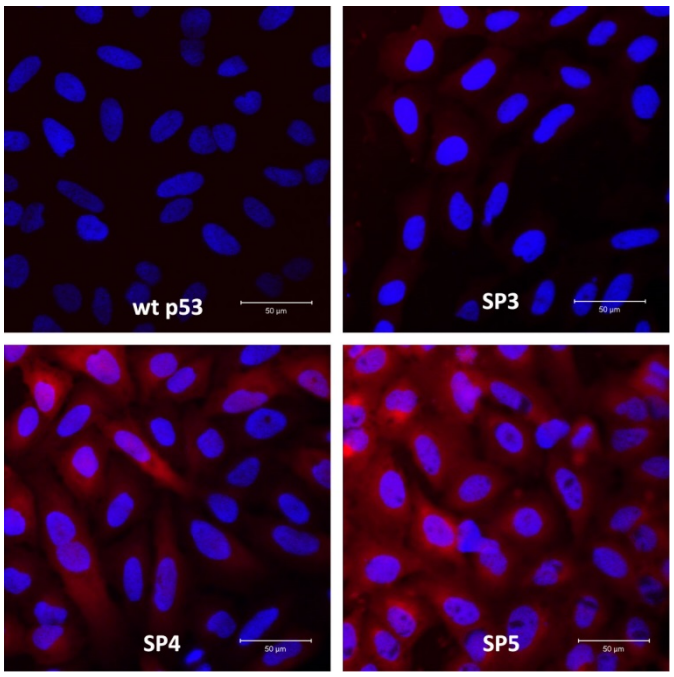

Figure 6. Confocal microscopy images of U2OS cells treated with 50 μM of TAMRA-labelled wild type $p53_{17-29}$, **SP3**, **SP4** and **SP5**. Nuclei are stained blue, whilst peptides appear in pink. Introducing positive charge on the linker induces cell permeability without changing the peptide sequence.

The unlabelled peptides were then tested in a T22 gene reporter assay[6b] to confirm whether the observed cellular uptake would correspond to the activation of p53 in cells. Indeed we observed a significant level of dose-dependent p53 activation upon treatment of cells with **SP5**, whilst minimal activation was observed in all other cases (Figure 7). We note that whilst TAMRA-labelled **SP4** (and to a lesser extent **SP3**) appear to enter cells by confocal microscopy, we do not observe significant activity in our gene reporter assay with unlabelled **SP3** or **SP4**. This may reflect low levels of uptake and the sub-cellular localisation of the stapled peptide. Appending the TAMRA dye itself also appears to affect properties such as peptide solubility, and issues involving uptake and the effect dye-labelling are currently the subject of further study. Finally, to confirm that both the staple and the cationic tag are necessary for cellular activity, we synthesised linear peptide **RRR-SP0**, which contains the three arginine cationic motif at the *N*-terminus of **SP0**, but missing the staple linker component. Compared with **SP5**, this control peptide had a reasonable affinity for MDM2 (Table 1), however was less helical (Supporting Information 4.5), and did not show any activation of p53 in the gene reporter assay. These results highlight the importance of the staple, in combination with the cationic motif, for achieving a cellular response.

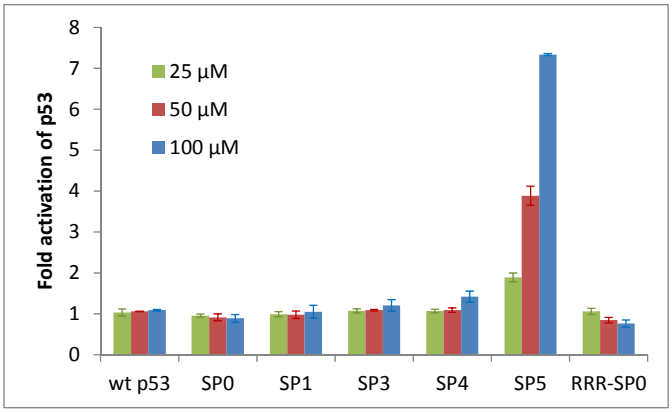

Figure 7. Fold activation of p53 in a T22 gene reporter assay after incubation with 25, 50 and 100 μM of peptides.

Conclusions

This proof of principle study demonstrates how our two-component stapling strategy enables the efficient optimisation of stapled peptide activity in cells. All five stapled peptide variants were synthesised in one step from the same unstapled peptide. We are now looking to gain a greater understanding of what factors are important for cellular activity by examining the cell-permeability of stapled peptides in greater detail, in particular the quantification of peptide uptake and localisation. We are also exploring alternative non-peptidic motifs which may confer cell permeability and activity. Finally, we will use our stapling chemistry together with peptide sequences optimised by phage display to efficiently explore more potent dual inhibitors of MDM2/MDMX with enhanced cellular activity.

Given the divergent nature, synthetic ease and functional group compatibility of this stapling methodology, we also envisage that other properties besides cell permeability could be tailored by designing an appropriate functionalised linker. Therefore we are exploring new staple structures and functional

motifs which have the potential to efficiently generate a vast array of chemical tools for enhancing our understanding of PPI networks and their inhibition.

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