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Investigating peptide sequence variations for 'double-click' stapled p53 peptides†

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Stapling peptides for inhibiting the p53/MDM2 interaction is a promising strategy for developing anti-cancer therapeutic leads. We evaluate double-click stapled peptides formed from p53-based diazidopeptides with different staple positions and azido amino acid side-chain lengths, determining the impact of these variations on MDM2 binding and cellular activity. We also demonstrate a K24R mutation, necessary for cellular activity in hydrocarbon-stapled p53 peptides, is not required for analogous 'double-click' peptides.

Peptide stapling is a side-chain macrocyclisation strategy for stabilising alpha-helical structure in short peptide sequences,[1] with the aim of generating inhibitors of protein–protein interactions.[2] Stapling typically involves introducing two alkenyl amino acids into a peptide sequence, followed by ring-closing metathesis to constrain the peptide in a helical conformation.[3] Pioneered by Grubbs, Verdine, Walensky and Sawyer, this hydrocarbon stapling approach can improve the binding affinity, proteolytic stability and *in vivo* activity of peptides, as demonstrated for a variety of protein–protein interactions.[4]

Complementary to the hydrocarbon stapling approach, there are a growing number of alternative stapling techniques which utilise different chemistries for peptide macrocyclisation.[5] These techniques can generate peptides with staples that are different from the standard hydrocarbon linkage, thereby potentially changing the overall properties of the peptide.[6] We have developed a double-click method of stapling peptides in solution,[7] where linear diazidopeptides are reacted with dialkynyl linkers to create bis-triazole stapled peptides under Cu(I) catalysis,[8] without the need for protecting groups (Fig. 1). As the peptide and linker are two separate components, this double-click methodology allows the properties of stapled peptides to be modified in two ways, either by introducing differently functionalised dialkynyl linkers or by modifying the peptide sequence itself.

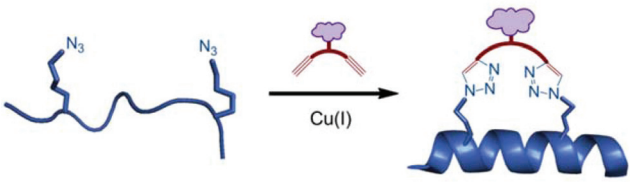

Fig. 1 Double-click peptide stapling – a linear diazidopeptide reacts with a dialkynyl linker under Cu(I) catalysis to give an alpha-helical peptide. Varying the linker functionality (in purple) and the peptide sequence (in blue) can change the biological activity of the overall stapled peptide.

In previous work,[7] we described the double-click stapling of a p53-based peptide for inhibiting the p53/MDM2 interaction, a promising target for cancer therapy. MDM2 is an E3 ubiquitin ligase overexpressed in some cancer cell lines, leading to loss of p53 function and subsequently uncontrolled abnormal cell growth.[9] Whilst we reported variations in the dialkynyl staple linkage, all of the stapled peptides were created from the same p53-based linear diazidopeptide **1** (Fig. 2). Herein, we report the synthesis and biological evaluation of double-click stapled p53 peptides based on a variety of different linear peptide precursors containing variations in the staple position and azido amino acid side-chain length, with the aim of understanding the impact of these variations on biological activity, and determining whether peptide **1** is indeed the optimal p53 diazidopeptide. Furthermore, we explore the effect of a single point mutation at residue 24, which has previously been suggested to be crucial for cellular activity.[10]

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Linker positions

1: Ac-ETFXDLWRLLXEN-NH_2
2: Ac-ETFSDXWRLLPEX-NH_2
3: Ac-ETFSXLWRLLPXN-NH_2
4: Ac-EXFSDLWRXLPEN-NH_2
5: Ac-XTFSDLWXLLPEN-NH_2
all X = Orn(N_3)

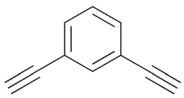
Side-chain length

Model peptides:
Ac-AAEAWAXAEAAEAXEA-NH_2
6: X = Orn(N_3)
7: X = Aha
8: X = Lys(N_3)

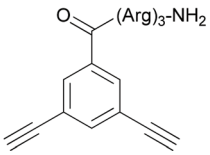
p53 peptides:
Ac-ETFXDLWRLLXEN-NH_2
9: X = Aha
10: X = Lys(N_3)

Lys/Arg-24 mutation

11: Ac-ETFXDLWKLLXEN-NH_2
where X = Orn(N_3)



A



B

Fig. 2 A range of diazidopeptides for stapling with dialkynyl linkers **A** and **B**.

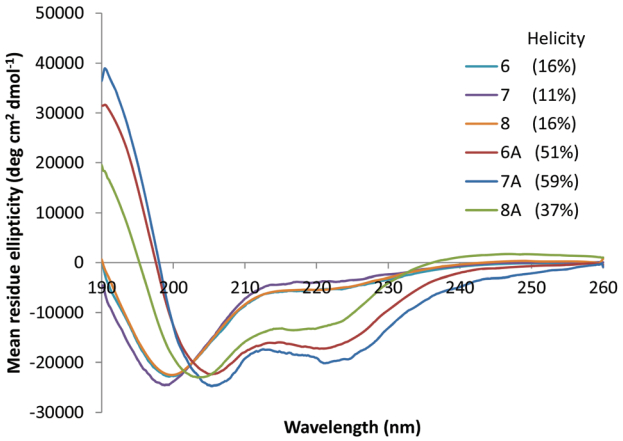


Fig. 3 Circular dichroism of model peptides **6–8** with different azido amino acid side-chain lengths, before and after stapling with linker **A**. Helicity is calculated from the mean residue ellipticity at 222 nm.

Table 1 Binding affinities of p53-based peptides **9**, **10** and **11** (stapled with linkers **A** and **B**), determined by competitive fluorescence polarisation, and compared with previously reported affinities for wild type $p53_{17-29}$, **1**, **1A** and **1B**[7]

Peptide	MDM2 K_i (nM)
wt $p53_{17-29}$	821 ± 56
1	16.1 ± 1.2
1A	3.21 ± 0.38
1B	11.7 ± 0.91
9	18.8 ± 1.5
9A	10.5 ± 0.76
9B	74.7 ± 4.8
10	9.34 ± 0.86
10A	9.63 ± 0.87
10B	33.9 ± 2.4
11	8.52 ± 0.80
11A	3.44 ± 0.46
11B	8.86 ± 0.82

Our initial choice of staple position in peptide **1** was based on analogous hydrocarbon stapled p53 peptides developed by Verdine,[10] with the two azido amino acids situated at residues 20 and 27. To investigate whether other i,i+7 staple positions could be used for double-click stapling, we synthesised four new linear diazidopeptides **2–5** (Fig. 2), placing azido amino acids at each possible i,i+7 pair, except for those which substitute one of three key amino acids Phe-19, Trp-23 and Leu-26 on the binding face of the peptide. After stapling with dialkynyl linker **A** however, none of the resulting stapled peptides **2A–5A** showed any appreciable binding to MDM2 (IC_{50} > 1000 nM) in our competitive fluorescence polarisation assay. Together with previous reports implicating Pro-27 replacement as a key driver of potency in p53-based peptides,[7,11] we concluded that the staple in peptide **1** is in the optimal position, as it is the only i,i+7 pair where Pro-27 is substituted.

We previously chose to use azido amino acid Orn(N_3) (three CH_2 units) in peptide **1** after studying Orn(N_3)-containing model peptide **6**.[7] Based on work by Inouye and coworkers,[5h] we previously found that this non-helical model diazidopeptide became helical upon stapling with linker **A**. To explore the effect of alternative azido amino acid side-chain lengths, we first synthesised model peptides **7** and **8**, containing the azido amino acids Aha (two CH_2 units) and Lys(N_3) (four CH_2 units) respectively. Upon stapling with linker **A** to give stapled peptides **7A** and **8A**, there was an increase in helical content in both cases, as determined by circular dichroism (Fig. 3). Comparing the three different side-chain lengths, Aha stapled peptide **7A** had the greatest overall helicity of 59%, compared to 51% for Orn(N_3) stapled peptide **6A**, whilst Lys(N_3) stapled peptide **8A** was only 37% helical. **7A** also had the largest percentage increase in helicity of 48% from linear to stapled peptide, compared to 35% for **6A** and 21% for **8A**.

With these model peptide results in hand, we synthesised linear p53 diazidopeptides **9** and **10**, which are the Aha and Lys(N_3) variants of peptide **1** respectively. In our competitive fluorescence polarisation assay (Table 1), both peptides **9** and **10** (18.8 ± 1.5 and 9.34 ± 0.86 nM respectively) displayed high affinity for MDM2 similar to that of peptide **1** (16.1 ± 1.2 nM), arising from the previously discussed Pro-27 replacement effect. Based on our model studies, we expected Aha peptide **9** to be optimal for stapling. Indeed, there was an approximately two-fold improvement in binding affinity when **9** was stapled with linker **A** to give **9A** (10.5 ± 0.76 nM), whilst stapling **10** to give **10A** (9.63 ± 0.87 nM) did not significantly change the binding affinity. However, Orn(N_3) stapled peptide **1A** (3.21 ± 0.38 nM) was still the most potent binder of MDM2, with the greatest increase in potency over its unstapled counterpart **1**. With linker **B**, a large reduction in potency was observed for

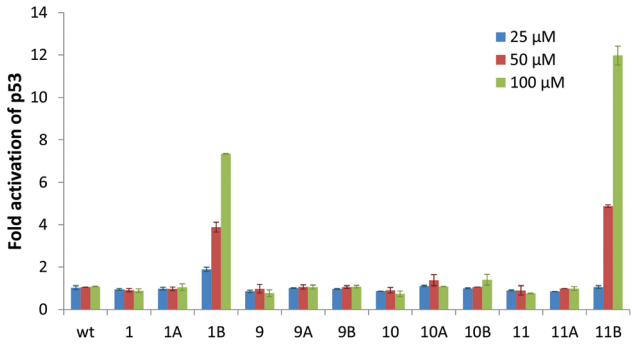


Fig. 4 Fold activation of p53 in a cell-based gene reporter assay. Lys-24 stapled peptide **11B** has a comparable activity to the Arg-24 stapled peptide **1B**.

9B (74.7 ± 4.8 nM) and to a lesser extent **10B** (33.9 ± 2.4 nM), whilst **1B** (11.7 ± 0.91 nM) showed the best affinity for MDM2. These results indicate that Orn(N_3) has the optimal side-chain length for stapling p53-based diazidopeptides, despite the model studies showing that Aha is better for generating helicity. The shorter side-chain of Aha appears to bring the staple too close to the peptide backbone, resulting in sub-optimal binding interactions with MDM2, whereas this is not apparent when only considering helicity of the model peptide in isolation. This effect is especially evident when stapling peptide **9** with bulkier linker **B** where a large potency drop is observed. We are now currently pursuing structural data to confirm these findings.

To determine whether the *in vitro* binding trends would be reflected in cells, we tested the peptides in a cell-based gene reporter assay. No p53 activation was observed for **9**, **10**, **9A** and **10A** up to 100 μM (Fig. 4), consistent with both our previous work where cationic staple **B** was required to achieve cellular activity with peptide **1**,[7] and work by Verdine where several negatively-charged residues in the p53 sequence were replaced with neutral amino acids.[10,12] Unlike cell-active stapled peptide **1B** however, cationic stapled peptides **9B** and **10B** showed little activity in the reporter assay, correlating well with their reduced binding affinity for MDM2. Therefore, the combination of *in vitro* and cellular assays reveals that the biological activity is significantly affected by changes in the azido amino acid, and that Orn(N_3) is the optimal azido amino acid for diazidopeptides based on the p53 sequence.

We then explored the effect of a point mutation at residue 24 in the sequence of peptide **1**. Although this residue is a lysine in the p53-wild type sequence, we previously chose to incorporate arginine at this position when designing peptide **1**. This mutation was again based on analogous hydrocarbon stapled p53 peptides,[10] where the Lys-24 variant found to be inactive in cells, potentially due to its native function as a ubiquitination site. To check whether incorporating Lys-24 would render our double-click stapled peptides inactive, we synthesised Lys-24 diazidopeptide **11** and generated the corresponding stapled peptides **11A** and **11B**. Stapled peptide **11A** was found to bind strongly to MDM2 *in vitro*, with a binding affinity similar to that of arginine-containing **1A** (Table 1). Cationic stapled **11B** had a slightly improved binding affinity over arginine-containing **1B**, and we observed significant dose-dependent activation of p53 in the cell-based assay (Fig. 4) comparable to that of **1B**. This result shows that using different stapling techniques (hydrocarbon *vs.* double-click) has a significant impact on the biological activity of a peptide, and highlights the ability of double-click stapling to endow a peptide with cellular activity *via* linker functionality, in this case obviating the need for further peptide sequence modifications.

In conclusion, we have synthesised and evaluated a collection of different p53-based diazidopeptides for double-click stapling. We confirm that Pro-27 replacement by the staple is important to achieve high affinity binding, and that Orn(N_3) has the most suitable side-chain length for achieving optimal binding and cellular activity. Furthermore, the biological activity is highly sensitive to the nature of the azido amino acid, with changes in the amino acid side-chain length not tolerated in our cell-based assay. We also find that stapled peptide **11B** with wild-type Lys-24 residue has comparable properties to Arg-24 containing peptide **1B**, indicating that this modification is not necessary for cellular activity in the case of double-click stapling. The knowledge gained from this study has important implications for the future design of double-click stapled peptides for inhibiting other protein–protein interactions, as it demonstrates that both the peptide sequence and the staple linkage are critical parameters which should be explored when optimising biological activity.

Acknowledgements

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