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Monosubstituted alkenyl amino acids for peptide "stapling"†

David J. Yeo,[ab] Stuart L. Warriner[ab] and Andrew J. Wilson*[ab]

Alkenylglycine amino acids were assessed as potential candidates for hydrocarbon stapling and shown to be effective in stapling of the BID BH3 peptide.

The elaboration of approaches to modulate protein–protein interactions (PPIs) is a highly sought after goal in contemporary chemical biology and drug discovery,[1,2] with significant effort focused on α-helix mediated PPIs.[3] In this regard, constrained peptides[4–9] have emerged as promising candidates for PPI inhibition given they are, in principle, able to reproduce exactly the pharmacophore of the template ligand, and have a range of enhanced properties. Introduction of constraints reduces the degree of freedom within a polypeptide backbone and if positioned correctly promotes the biologically active conformation and more potent binding to target receptors. Typically, constraining a peptide confers greater resilience to proteolytic digestion, since a peptide must unfold in order to fit into a protease active site,[10] and, in a number of instances has been shown to assist cellular uptake and activity.[11] Synthetic approaches include functionalisation of peptides with azide–alkyne 'click' handles,[12] lactam bridges,[7] hydrogen bond surrogates[13] and metathesis derived hydrocarbon 'staples'. All-carbon hydrocarbon stapling, pioneered by Grubbs[14] and Verdine,[15] is continuing to grow in popularity. During peptide synthesis, an unnatural α,α-disubstituted amino acid with an alkene tether is incorporated at *i*, *i* + 4, or *i*, *i* + 7 positions and metathesised catalytically affording a covalent crosslink on one face of the peptide.[15–17] Following evaluation of cross-link length, positioning and helix-stabilising propensities using RNAseA,[15] the use of hydrocarbon stapling for inhibition of PPIs within the B-cell lymphoma (BCL-2) family,[18–20] the NOTCH pathway,[21] p53–*h*DM2(X) interactions[11,22] caspase PPIs[23] and estrogen receptor–co-activator interactions[24] has been described. Recently, a stapled peptide has entered phase I clinical trials.[25] In the course of our ongoing efforts to develop inhibitors of PPIs using α-helix mimetics,[26] we became interested in making comparative studies with other ligand classes and sought to develop "stapled" peptides. Our initial efforts relied upon synthesis of the requisite α,α′-disubstituted alkenyl amino acids – a synthetic sequence which we found to be temperamental in our hands. Thus we asked the question – can similar effects be achieved with monosubstituted α-alkenyl amino acids? Herein we illustrate that such a modification indeed serves as an effective staple and illustrate that multiple biophysical properties are comparable to peptides stapled with α,α′-disubstituted amino acids.

Given our prior work,[26] we initially sought to test the monosubstituted amino acid as a constraint using p53–*h*DM2 as a model PPI, however initial attempts to select suitable positions in which to incorporate a staple afforded a peptide with lower binding than the unstapled peptide (see ESI†); we were not entirely surprised by this given the extensive work by the Verdine group on identifying optimal stereochemistry and chain length of the disubstituted amino acids for introduction of peptide staples at different positions in a sequence.[15,17] We therefore resorted to the BCL-2 family of PPIs as a model system (Fig. 1a), following the prior work on *in vivo* apoptosis of leukaemia xenografts using hydrocarbon stapled BID BH3 peptides.[18] The BID BH3 peptide is a region of the pro-apoptotic BID protein which belongs to the family of BCL-2 proteins that regulate apoptotic pathways.[27–30] The BH3 region of BID is an amphiphilic α-helix, which binds into a hydrophobic groove within the multidomain anti-apoptotic members of BCL-2 family members.[31] The synthesis of (*S*)-pentenylglycine has previously been described[32] following the method developed by Belokon and co-workers for synthesis of non-natural amino acids.[33] Our group have successfully utilised this method in other work[34] and applied it here (Fig. 1b and ESI†). For a full comparison, we also synthesized the original disubstituted amino acid (see ESI†)[15] and incorporated this, the monosubstituted amino acid and aminoisobutyric acid (Aib) at appropriate positions in the BID sequence which were obtained using standard Fmoc solid phase peptide synthesis protocols using Rink Amide MBHA resin to afford C-terminally amidated peptides (Fig. 1c). Alkenyl amino acid containing peptides were subjected to olefin metathesis to generate stapled peptides with the un-metathesised variants retained as controls (not shown, see ESI† for details).

[a] *School of Chemistry, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK. E-mail: A.J.Wilson@leeds.ac.uk; Fax: +44 (0)113 3436565; Tel: +44 (0)113 3431409*
[b] *Astbury Centre for Structural Molecular Biology, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK*

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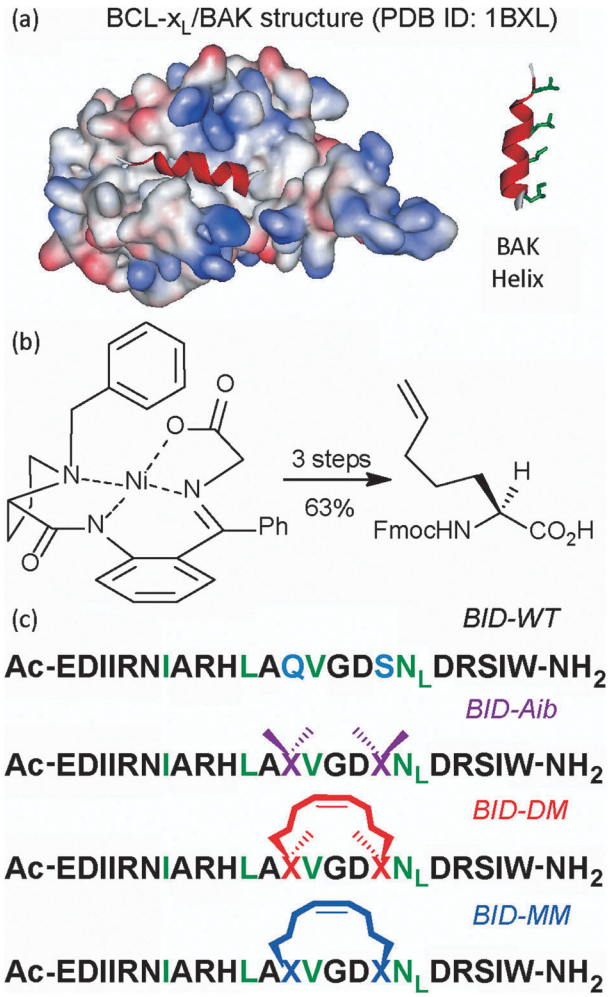

Fig. 1 (a) Structure of the BCL-x_L in complex with a BAK peptide (PDB-ID:1 BXL); (b) abbreviated synthetic route to α-pentenyl glycine (c) sequence of the BID BH3 peptides, BID-WT, BID-Aib, BID-DM and BID-MM. (N_L = norleucine substitution for wild type Met)[18]

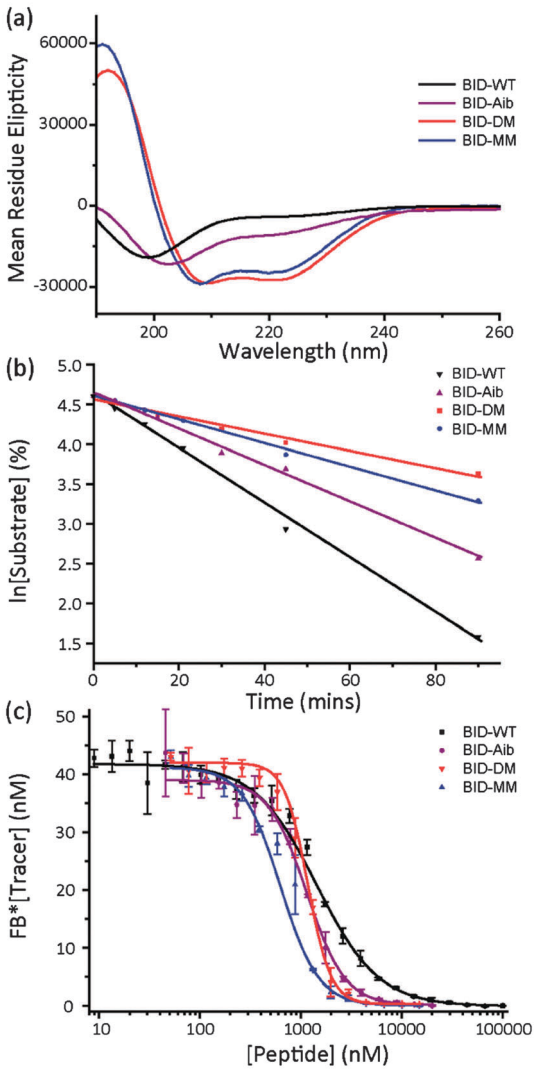

Fig. 2 Biophysical data for the BID peptides; BID-WT (black), BID-AIB (purple), BID-DM (red), BID-MM (blue), (a) circular dichroism shows enhanced helicity after stapling; (b) trypsin proteolysis studies. (c) Fluorescence anisotropy competition assays for the modified peptides.

The helix stabilising properties of each mutation to the sequence were assessed using circular dichroism (Fig. 2a). The spectra clearly illustrate that the monosubstituted and disubstituted amino acids promote helicity with minima at 208 and 222 nm, compared to a random coil for the wild type and intermediate level of helicity for the Aib substituted sequence. BID-WT, BID-Aib, BID-DM, BID-MM, gave α-helicities of 12%, 31%, 80% and 73% respectively. With this result in hand, enzymatic degradation experiments with trypsin were performed using HPLC to determine the extent of resistance to proteolysis upon stapling. Trypsin selectively hydrolyses the peptide bond to the C-terminal side of basic residues, lysine and arginine, of which three are present in the BID BH3 sequence. HPLC analysis showed that the full peptide was degraded into smaller fragments during the experiment, and plotting ln S against time indicated first order kinetics with respect to the substrate (Fig. 2b). BID-WT, BID-Aib, BID-DM, BID-MM were found to be 5%, 12%, 38% and 35% intact respectively after 90 minutes, indicating that stapling the peptide confers significant resistance to proteolytic degradation, but again that little difference exists between the monosubstituted and disubstituted amino acid staples. Finally, a consequence of restricting the conformational plasticity of a peptide is an increase in binding affinity of the peptide to its protein partner. To test the binding capabilities of the peptides, we used a fluorescence anisotropy based competition assay to follow the displacement of BODIPY labelled BAK from the BH3 binding groove of BCL-x_L. Briefly, a BODIPY labelled BAK was shown in our hands to exhibit a Kd of 4 nM towards BCL-x_L in the forward titration experiment (see ESI†). Displacement of this tracer peptide under appropriate conditions afforded IC_{50}'s as shown in Fig. 2c. Curiously, none of the modifications result in significant improvement in inhibitory potency (IC_{50}'s for BID-WT, BID-Aib, BID-DM, BID-MM were found to be 1.44 (±0.05), 1.14 (±0.02), 1.15 (±0.04) and 0.62 (±0.02) μM). The Hill coefficients in the curves for both stapled peptides are noteworthy and point to a more complex equilibrium than a standard 1 : 1 competition. This does not arise due to interaction between the peptide tracer (BODIPY-BAK) and the competitor peptides (as we observed previously for a p53–*h*DM2 anisotropy assay)[35] as we did not detect changes in anisotropy upon titration of BODIPY-BAK with

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BID peptides. More detailed biophysical and structural studies are in progress.

A significant feature of the stapling approach is the supposed enhanced cellular uptake conferred upon a peptide as a result of stapling, however recent evidence has indicated that this is a complex issue with experimental conditions playing a significant role.[36] Similarly in certain instances, whilst stapling does not appear to improve potency or the propensity of a peptide to adopt its bioactive conformation in comparison to other modifications, it can result in improved *in cellulo* PPI inhibition.[11] The current preliminary results thus contribute to the growing body of research on peptide stapling; from a biophysical perspective, a modification to any given peptide sequence *e.g.* the use of β-amino acids[37,38] which tips the balance in favour of helix nucleation[39,40] is what is required for enhanced proteolytic stability and a preferred bioactive conformation. In contrast, cell uptake and function need to be considered and optimised separately.[41]

In summary, we have shown that α-alkenyl substituted amino acids act as effective amino acids for peptide stapling and demonstrate that they can lead to comparable enhancement of proteolytic stability, enhancement of helical propensity and similar efficiency compared to peptides stapled with an α,α′-disubstituted variant when applied to the BID BH3 helix and its inhibition of BCL-x_L/BAK as a model PPI. This modified amino acid is less sterically encumbered than disubstituted amino acids and may facilitate synthesis and therefore studies of stapled peptides. Our future work will focus on structural and biophysical measurements on this and other sequences in addition to more systematic studies on cellular behaviour of constrained peptides.

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