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COMMUNICATION

Recognition of ASF1 Using Hydrocarbon Constrained Peptides

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Abstract: Inhibition of the histone H3-ASF1 (anti-silencing function 1) protein-protein interaction (PPI) represents a potential approach for treatment of numerous cancers. As an α-helix mediated PPI, constraint of the key histone H3 helix (residues 118-135) represents a strategy through which chemical probes might be elaborated to test this hypothesis. In this work variant $H3_{118-135}$ peptides bearing pentenyl glycine residues at i and i + 4 positions were constrained by olefin metathesis. Biophysical analyses revealed that promotion of a bioactive helical conformation depends on the position at which the constraint is introduced, but that potency of binding towards ASF1 is unaffected by the constraint and instead that enthalpy-entropy compensation occurs.

A significant unmet goal in chemical biology is to develop methods for inhibition of protein-protein interactions (PPIs).[1-2] In the context of α-helix mediated PPIs,[3] considerable effort has been exerted in developing methods for constraining (or "stapling") peptides in an α-helical conformation. This approach has been used to confer enhanced proteolytic stability, enhanced cell-uptake, and, in some cases enhanced target affinity of constrained peptide sequences.[4-23] We recently introduced a series of reagents and approaches for constraining peptides in a helical conformation.[24-27] Of these, use of variant peptides bearing alkenyl glycine residues in the i and i + 4 positions constrained through olefin metathesis was previously shown to be effective in biasing the sequences of variant BCL-2 BH3 sequences towards the helical conformation.[27] Subsequently we demonstrated these peptides bind to their target BCL-2 proteins through an induced fit mechanism but do not elicit enhanced target affinity arising from enthalpy-entropy compensation as demonstrated by SPR and van't Hoff analyses respectively.[26] Herein, using the anti-silencing function 1 (ASF1) chaperone as a protein target, we demonstrate the broader applicability of S-pentenyl-glycine variant peptides as substrates for hydrocarbon constraining and further reinforce the notion that constraining the peptide in a bioactive conformation may not lead to increased affinity for the target protein as a consequence of enthalpy-entropy compensation.

Histone chaperones regulate the association of basic histone proteins with DNA, permitting nucleosome assembly in an ordered and controlled manner.[28-33] Histone chaperone anti-silencing function 1 (ASF1) is a highly conserved histone chaperone involved in both Histone H3-H4 handling and buffering.[34-38] It has been shown to play a key role in development and progression of some cancers, hence represents a potential target for chemical probe and drug discovery.[39-41] The interaction between ASF1 and the H3 and H4 histone proteins forms a ASF1-(H3-H4) complex preventing the formation of the histone H3-H4 tetramer and shielding H3-H4 dimers from unfavorable interactions. The reestablishment of the tetramer was proposed to be the key element for the formation of the nucleosome (Fig. 1a).[42] The ASF1 protein comprises a conserved N-terminal domain of 156 amino acids, which is essential for its function in vivo, and a divergent unstructured C-terminal domain, which is not considered necessary for function.[37, 43] The structure of the ASF1 comprises an elongated β sandwich core with three α-helices in the loops between the β strands (Fig. 1b). The contacts between H3 and ASF1 are extensive and result in a buried surface area of 909 Å^2. The histone H3 binding site is located in the concave face of ASF1 (Fig. 1b) and involves β strands β3, β4, and β6-9.[37, 43-44] The main interactions occur through the C-terminal helix of H3 (residues 122–134), where the key residues Leu126 and Ile130 form a hydrophobic clamp with the hydrophobic region of ASF1. Additionally, there is a network of electrostatic interactions within the PPI interface, such as the salt bridge between Arg129 from H3 and Asp54 from Asf1.[45] The ASF1-H3-H4 structure also shows extensive contacts between ASF1 and histone H4 [44] and has two parts (not shown): the globular core of ASF1 interacts with the C-terminal tail of H4 to form a strand-swapped dimer and the C-terminal tail of ASF1 binds to the histone fold region of histone H4.

We envisioned the C-terminal α-helix peptide of H3 as a template for the design of molecules able to recognize ASF1. We used S-pentenylglycine rather than S-pentenylalanine as the former is easier to synthesize and demonstrates comparable behavior in biophysical analyses.[27] The sites to incorporate the mono-alkenyl substituted amino acids within the peptide sequence were selected taking into account: (i) the requirement to appropriately position the non-natural amino acids so as to constrain in a manner that promotes a helical conformation (i.e. i and i+4 positions); (ii) the need to position the hydrocarbon bridge so as not to sterically occlude "wild-type" interactions necessary for recognition. On this basis, two options were considered Met120/Ile124 and Asp123/Ala127. **$H3_{118-135}$**, together with variants bearing S-pentenyl-glycine in the identified positions were prepared by solid-phase peptide synthesis (see ESI) and the later cross-linked by olefin metathesis to give **$H3_{118-135(St120-124)}$** and **$H3_{118-135(St123-127)}$GCA** (the GCA sequence was introduced for future functionalization e.g. cell-penetrating sequences,

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Supporting information for this article comprising synthetic procedures, details of protein expression, biophysical and NMR analyses is given via a link at the end of the document.

fluorophores etc. via the nucleophilic thiol of the cysteine residue). On resin ring closure proceeded quantitatively in 4 hr.

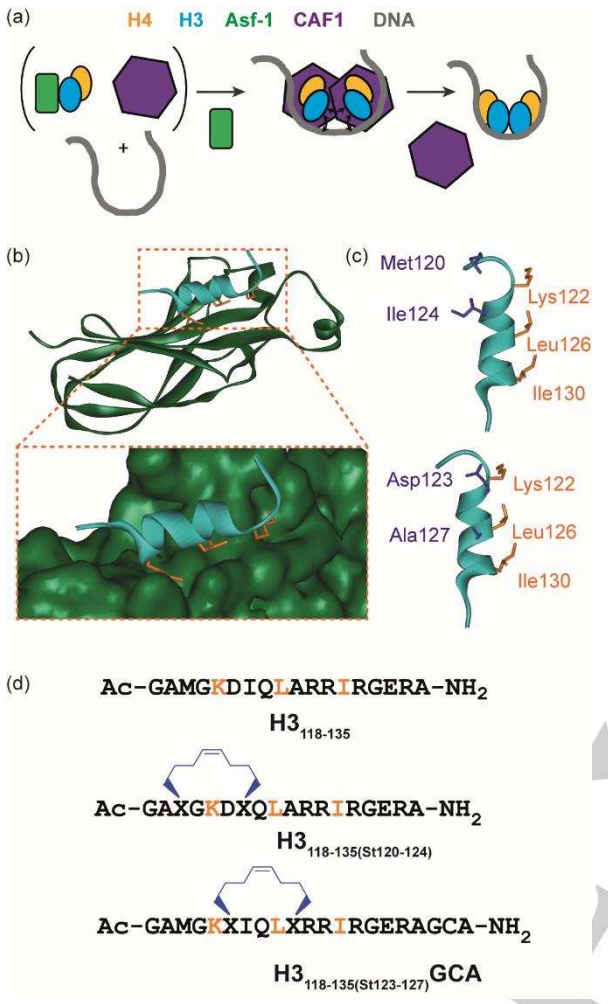

Figure 1. ASF1 as a target for constrained peptides (a) schematic illustrating the role of anti-silencing function 1 (ASF1 in green) in displacement of CAF-1 (purple) through recognition of Histone H3 (cyan) and H4 (yellow) so as to facilitate nucleosome formation (b) structure of the Histone H3(118-135) (cyan)-ASF1A(1-156) (forest green) interaction as determined by NMR (PDB ID: 2IIJ)[45] – Histone side chains located on-one face perceived to be important for binding are shown as orange sticks (c) key H3 helix (cyan), highlighting key side chains (orange) and residues at i, i + 4 positions considered suitable for introduction of a constraint (top: M120/I124, bottom: D123/A127), (d) sequences of peptides used in this studied highlighting position of hydrocarbon constraint.

The helical character of all three peptides was investigated using circular dichroism (CD) in both 40 mM phosphate buffer and trifluoroethanol (TFE). In aqueous solvent **H3$_{118-135}$** and **H3$_{118-135(St120-124)}$** both exhibited CD spectra consistent with a predominantly random coil conformation (% helicities **H3$_{118-135}$** = 15% and **H3$_{118-135(St120-124)}$** = 20%), whilst in the presence of the helix promoting TFE (see ESI)[46-47] the CD spectra were indicative of a more α-helical signature indicating both possess sufficient conformational flexibility to access the helical conformation required for specific ASF1 binding. It is perhaps unsurprising that constraining the peptide between residues 120 and 124 did not promote a helical conformation in **H3$_{118-135(St120-124)}$** given the observation from the H3/ASF1 NMR structure that the H3 helix is distorted/frayed at the N-terminus close to M120. In contrast, **H3$_{118-135(St123-127)}$GCA** was shown to adopt, as expected, a more helical conformation in aqueous solution by CD analyses (% helicity = 29%). The data for all three peptides in TFE (see supporting information) demonstrate that each of the peptides is capable of adopting a helical conformation to a comparable extent, and, that there is little difference between buffer and TFE for **H3$_{118-135(St123-127)}$GCA**, indicating the sequence has intrinsically low helical propensity.

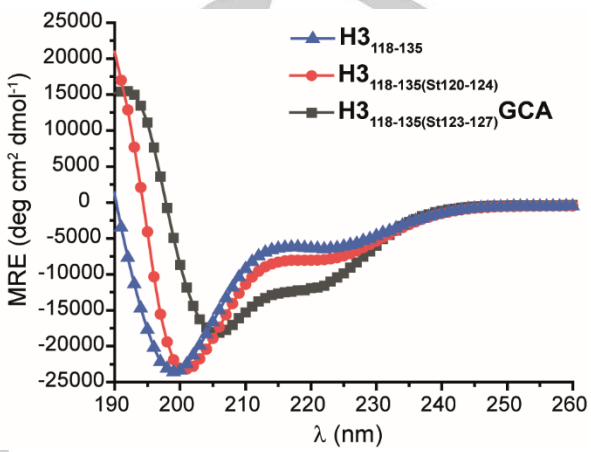

Figure 2. Conformational analyses of histone H3 variant peptides by circular dichroism (CD) analyses (peptide concentration =100 μM, 40 mM sodium phosphate, 293K, pH7.5)

Binding of the peptides to ASF1 was then assessed using isothermal titration calorimetry (Fig. 3, Table 1). All three peptides exhibited exothermic binding and could be fit to a 1:1 binding isotherm. Strikingly the binding potency was similar in all three cases ΔG = -7.3 to -8.0 kCal M^{-1} despite **H3$_{118-135(St123-127)}$GCA** adopting a more helical conformation and therefore presumably being more pre-organized towards ASF1 recognition. Analyses of the thermodynamic determinants of binding reveal enthalpy-entropy compensation. Both **H3$_{118-135}$** and **H3$_{118-135(St120-124)}$** exhibited favorable enthalpies of binding (ΔH = -14.4 to -15.0 kCal M^{-1}) but the binding entropies were unfavorable (TΔS = -6.4 to -6.7 kCal M^{-1}). In contrast, for the more helical peptide, **H3$_{118-135(St123-127)}$GCA** the entropy of binding (TΔS = 3.1 kCal M^{-1}) was favorable consistent with the anticipated effect of pre-organization, however the enthalpy of binding (ΔH = -4.2 kCal M^{-1}) was less favorable than for the less helical variants. It is noteworthy that **H3$_{118-135(St120-124)}$** and **H3$_{118-135(St123-127)}$GCA** despite both containing a staple; the former is less helical and exhibits a large favorable enthalpy change with unfavorable entropy, whereas the later is more helical and has a less favorable enthalpy of binding but more favorable entropy of binding. Such an effect may arise because the less helical peptides **H3$_{118-135}$** and **H3$_{118-135(St120-124)}$** form enthalpically favorable backbone hydrogen-bonds upon a change in conformation to the helix, whereas the more pre-organized helix **H3$_{118-135(St123-127)}$GCA** neither gains new hydrogen-bonds nor undergoes the entropically costly change in conformation on binding ASF1. Alternatively differential changes in solvation of the peptides upon binding may account for such a difference in thermodynamic signature. Either way, the results underscore a limitation in correlating conformational stability

against binding potency for the unconstrained (**H3$_{118-135}$**) and constrained (**H3$_{118-135(St123-127)}$GCA**) peptides; although the helical conformation is preferred for **H3$_{118-135(St123-127)}$GCA** this can be considered as arising due to an increase in energy (or destabilization) of non-helical conformations for this sequence as opposed to preorganization of the wild-type sequence.

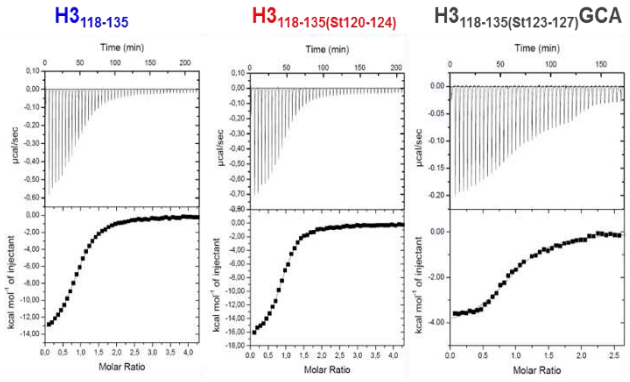

Figure 3. ITC thermograms and data fitting for the interaction of **H3$_{118-135}$** (left) **H3$_{118-135(St120-124)}$** (centre) and **H3$_{118-135(St123-127)}$GCA** (right) with ASF1A(1-156).

Table 1. Thermodynamic parameters for the binding of histone H3 peptide variants to ASF1 as determined by Isothermal titration calorimetry (see Fig. 3 for details)

Peptide	K_d (µM)	ΔG (kCal/)	N*	ΔH (kCal/M)	TΔS (kCal/M)
H3$_{118-135}$	1.34 ±0.33	-8.0 ±0.14	0.94 ±0.04	-14.4 ±0.37	-6.4 ±0.51
H3$_{118-135(St120-124)}$	0.86 ±0.11	-8.3 ±0.07	0.97 ±0.01	-15 ±0.96	-6.7 ±1.03
H3$_{118-135(St123-127)}$GCA	1.6 ±0.13	-7.3 ±0.05	0.97 ±0.01	-4.2 ±0.08	3.1 ±0.2

In order to confirm the binding mode of the constrained peptides with ASF1, chemical shift perturbation studies were carried out for all three peptides (see ESI) using uniformly ^{15}N labelled ASF1A(1-156). The chemical shift variation was mapped onto the protein structure of ASF1A-H3 (PDB ID: 2IIJ). All three peptides induced the highest values of chemical shift variations and a "slow exchange" regime for ASF1 residues defining the histone already well characterized H3 binding site (V_{45}-E_{51}, V_{90}-I_{97}, R_{108}-Y_{111}, V_{146}-T_{147}),[37, 45] confirming the preservation of the specific binding mode for the constrained peptides. In addition, both **H3$_{118-135}$** and **H3$_{118-135(St120-124)}$** exhibited chemical shift variations on the opposite side of the protein surface corresponding to the B domain binding site (S_{59}-F_{72}),[48] that most probably corresponds to nonspecific binding in the case of the histone peptide. Interestingly, constrained **H3$_{118-135(St123-127)}$GCA** induced no chemical shift variation in this region of the ASF1 (see ESI). This result suggests that unfolding of the helical conformation is probably required for this nonspecific binding.

Proteolytic stability of the peptides was also investigated using trypsin and proteinase-K. The unconstrained **H3$_{118-135}$** was cleaved within 14 minutes by both proteases (Fig. 4, Table 2 and ESI), whereas the constrained peptides resulted in increased stability depending on the position of the constraint. **H3$_{118-135(St120-124)}$** was less susceptible to cleavage by proteinase K ($t_{1/2}$ = 65.8 min), on the other hand **H3$_{118-135(St123-127)}$GCA** showed increased stability against trypsin ($t_{1/2}$= 40.5 min). The constraint also affected the profile of cleavage sites, most notably for **H3$_{118-135(St123-127)}$GCA** where, two proteinase-K cleavage sites where suppressed by introduction of the constraint. The results indicate the need to consider the results of proteolytic cleavage studies on constrained peptides, as the protective effect likely arises not only due to the enhanced helicity i.e. the greatest effect is observed for the constraint that does not markedly promote helicity (**H3$_{118-135(St120-124)}$**).

Table 2. Fitted half-lives of the peptides in the presence of proteases.

Peptide	Trypsin $t_{1/2}$ (min)	Proteinase K $t_{1/2}$ (min)
H3$_{118-135}$	13.3 ± 1.5	12.2 ± 0.8
H3$_{118-135(St120-124)}$	14.9 ± 2.5	65.8 ± 15.7
H3$_{118-135(St123-127)}$GCA	40.5 ± 16.9	23.1 ± 2.6

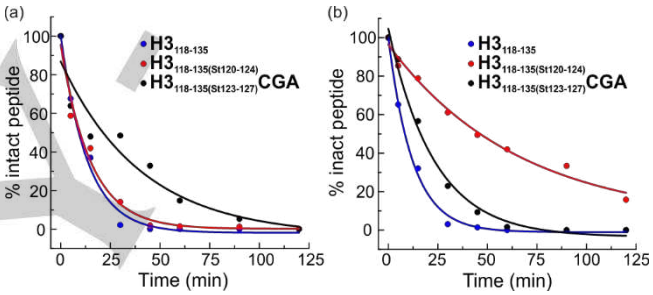

Figure 4. Proteolytic stability of the peptides against (a) trypsin and (b) proteinase K.

In conclusion, we have shown that variant H3$_{118-135}$ peptides bearing pentenyl glycine residues at i and i + 4 positions can be constrained by olefin metathesis to generate a peptide more biased towards a helical conformation than the parent wild-type sequence. This further broadens the scope of this non-natural amino acid for hydrocarbon "stapling". In addition, we have illustrated that a more helical conformation (i.e. for **H3$_{118-135(St123-127)}$GCA** does not necessarily correlate with significant proteolytic protection or enhanced binding potency and that here the later aspect is concerned, instead enthalpy-entropy compensation is observed. Nonetheless, constraining peptides has been shown to reduce nonspecific binding and to enhance a range of additional pharmacokinetic properties e.g. cell-uptake. The peptide sequence used in this work was shown to have moderate helical propensity, thus our future studies will center on exploiting the constraining strategy together with helix stabilizing amino acids to optimize these reagents for binding and cell permeability to develop chemical probes of the H3-ASF1 interaction.

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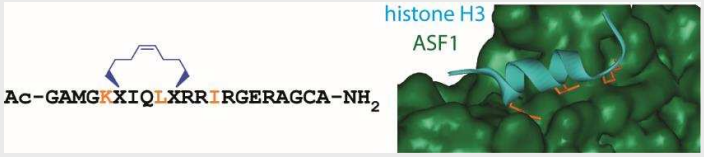

Binding affinity of hydrocarbon constrained Histone H3 peptides towards Anti-silencing Function 1 (ASF1) is shown to be unaffected by pre-organisation and instead enthalpy-entropy compensation occurs

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