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N terminal *N*-methylation modulates chiral centre induced helical (CIH) peptides' biophysical properties†

Wenjun Li,‡[a] Kuan Hu,‡[bc] Qingzhou Zhang,[a] Dongyuan Wang,[a] Yue Ma,[a] Zhanfeng Hou,[a] Feng Yin*[a] and Zigang Li*[a]

The effects of *N*-methylation on CIH peptides' biophysical properties were systematically studied. *N*-Methylation at the N terminal NH could help improve the peptides' cellular uptake with a retained helical conformation. This *N*-methylation strategy could also be applied to longer peptides to improve their stability and cellular uptake.

N-Methylation is prevalent in natural peptides such as cyclosporine.[1] It blocks hydrogen-bond donors to decrease the solvent–peptide interactions, increases the hydrophobicity of peptides and strengthens the peptide backbone hindrance, thus, *N*-methylation at suitable positions may help the peptides to obtain desirable pharmacokinetic profiles including better serum stability and cellular uptake.[2–8] Continuous efforts have been invested by the research community, such as Kessler *et al.* who utilized multiple *N*-methylation strategies with the MT-II peptide to target hMC1R[4] and Miller *et al.* who developed efficient *N*-methylated cyclic peptide ligands targeting various proteins and RNAs.[5] In addition to *N*-methylation, substituents other than methyl were also reported to tune peptides' biophysical properties, such as Barron *et al.*'s report on *N*-alkylated peptide mimetics of Magainin-2.[7]

Meanwhile, stabilizing peptides with an artificial crosslink is also a well-studied method to fix the peptides into a regulated α-helix or β-sheet structure for preferable biophysical properties.[8] Recently, our group developed an in-tether chiral-centre-induced helicity (CIH) strategy to stabilize peptides into an α-helix structure. In the CIH strategy, an in-tether chiral centre at the γ position to the peptide's C-terminus (C-γ) would dominate the peptide's secondary structure as shown in Fig. 1.[9] Only the *R* epimer is helical while the *S* epimer is mainly random coil. Further research indicated that the chiral centre could be utilized to tune the peptides' biophysical properties.[10] In our previous study, we carefully elucidated the intra-helical hydrogen bond network *via* X-ray crystal structure analysis and 2D NMR study. In this report, we systematically studied *N*-methylation at different positions of CIH peptides, for the influence on both their secondary structures and other related biophysical properties. Based on the best of our knowledge, the study on *N*-methylation of constrained peptides is still sporadic. This report is the first attempt to systematically elucidate the importance of maintaining the intra-helical hydrogen bond network of constrained peptides, and to identify the N terminal NH as the suitable methylation site for improving CIH peptides' biophysical properties. After the proof-of-concept study on the pentapeptide model system, we further elucidated the advantages of modification in constructing longer peptides targeting the HIF-1α transcription coactivator interaction.[11]

Therefore, we synthesized a panel of simplified model pentapeptides as shown in Fig. 2A. Peptide **1*R*** is a pentapeptide without *N*-methylation and *R* denotes the absolute configuration of the in-tether chiral centre. For all CIH peptides used in this

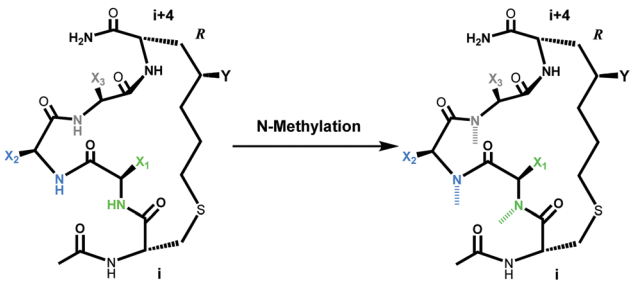

Fig. 1 The schematic presentation of possible *N*-methylation sites of CIH peptides, marked with dashed bonds. X_1, X_2, and X_3 are amino acids, Y is the substituent at the in-tether chiral centre, and **R** indicates the absolute configuration of the chiral centre.

[a] *The Key Laboratory of Chemical Genomics School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, China 518055. E-mail: yinfeng@pkusz.edu.cn, lizg@pkusz.edu.cn*
[b] *Beijing Institute of Nanoenergy and Nanosystems, National Center for Nanoscience and Technology (NCNST), Chinese Academy of Sciences, Beijing 100083, China*
[c] *College of Nanoscience and Technology, University of Chinese Academy of Sciences, Beijing 100049, P. R. China*

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‡ These authors contributed equally to this work.

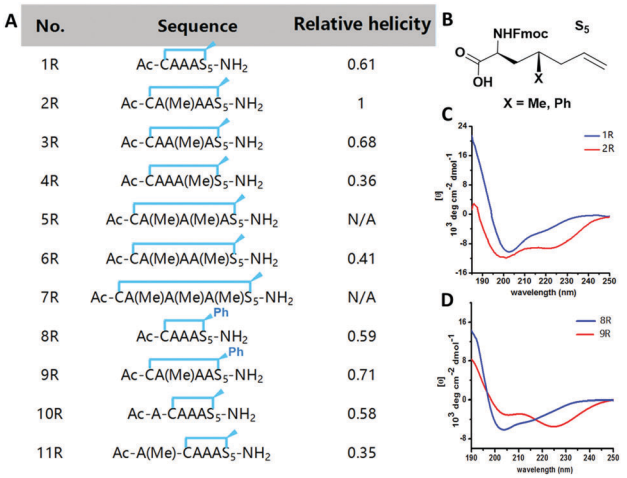

Fig. 2 N-Terminus methylation maintains the peptide's helical conformation. (A) Sequences and relative helicity of CIH peptides with methylation at different places. As peptides **5*R*** and **7*R*** have positive θ_{215} values on their CD spectra, thus their helical contents are denoted as N/A. The α-helical content of each peptide was calculated using the reported formula,[12] with the helicity of peptide **2*R*** fixed at 100%. (B) The chemical structure of unnatural amino acid S_5, X is methyl or phenyl. (C) CD spectra of CIH peptide **1*R*** and its *N*-methylated analogue **2*R*** in ddH_2O at 298.15 K. (D) CD spectra of peptide **8*R*** and its *N*-methylated analogue **9*R*** in ddH_2O at 298.15 K.

study, their in-tether chiral centres were pre-determined to be *R* using enantiomerically pure unnatural amino acids.[10] Peptides **2*R*–4*R*** were prepared through *N*-methylation at different positions for intra-helix hydrogen bond masking. Notably, circular dichroism (CD) spectroscopy analysis showed that peptide **2*R*** maintained some features of helical structures (Fig. 2C), while peptides **3*R*** and **4*R*** showed significantly decreased helical contents as shown in Fig. S1 (ESI†). Then peptides **5*R*–7*R*** with multi *N*-methylation were prepared and their secondary structures were analysed by CD spectroscopy. As we expected, their helical features were basically diminished as shown in Fig. S2 (ESI†). These results indicated that for the CIH pentapeptide model system, masking N-terminus NH which does not take part in the intra-helix hydrogen bond network will maintain helical features, while masking H-bond participating NH at other positions (methylated positions shown in peptides **3*R*** and **4*R*** respectively) will largely disrupt or diminish the peptides' helical structures.

Then the substituent on the in-tether chiral centre was switched from methyl to phenyl, as shown in Fig. 2D to make peptides **8*R*** and **9*R***. They behaved similarly in their CD measurements to peptides **1*R*** and **2*R*** (Fig. 2C). Thus, this model study revealed an interesting fact: the intra-helical H-bond network is still essential for constrained peptides, although it is supposed to be fixed by artificial crosslinks into helical structures. The intra-helical H-bond network and the artificial linker play synergistic roles in maintaining the helical structure. To further explore the influence of *N*-methylation at positions outside the nucleating core of constrained peptides, peptides **10*R*** and **11*R*** were prepared. And as we expected, peptide **11*R*** with *N*-methylation outside the ring region showed a reduced helical content as shown in Fig. S3 (ESI†). However, peptide **11*R*** showed a relatively higher

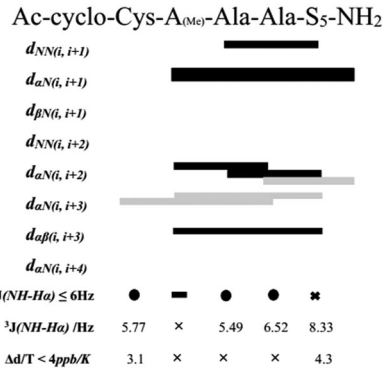

Fig. 3 NOE summary diagram of peptide **2*R*** (measured in 10% D_2O in H_2O, 298.15 K). Bar thickness parallels the intensity of the NOE signals.

cellular uptake than **10*R*** (Fig. S5, ESI†) which triggered our further investigation.

Detailed 1D and 2D ^{1}H-NMR studies on peptide **2*R*** were performed in 10% D_2O in H_2O at 298.15 K. A number of α-helical features can be observed from the ^{1}H NMR spectrum. Low coupling constants ($^3J_{NHCH\alpha} < 6$ Hz) for most of the amide resonances except for the C terminal residue suggest an α-helical structure of this pentapeptide, as shown in Fig. 3. In addition, the signals in the NOESY spectra of non-sequential medium long range: $d_{\alpha N}(i, i+3)$, $d_{\alpha\beta}(i, i+3)$, and $d_{\alpha N}(i, i+4)$ NOESY also indicate a helical structure. The low $\Delta\delta/T$ (less than 4.5 ppb K^{-1}) in the temperature coefficient assay for the backbone amide NH chemical shift also demonstrates the helical features of peptide **2*R*** (Fig. S6, ESI†). In summary, both the NOE and CD spectra of peptide **2*R*** suggest that it maintains a helical structure in solution.

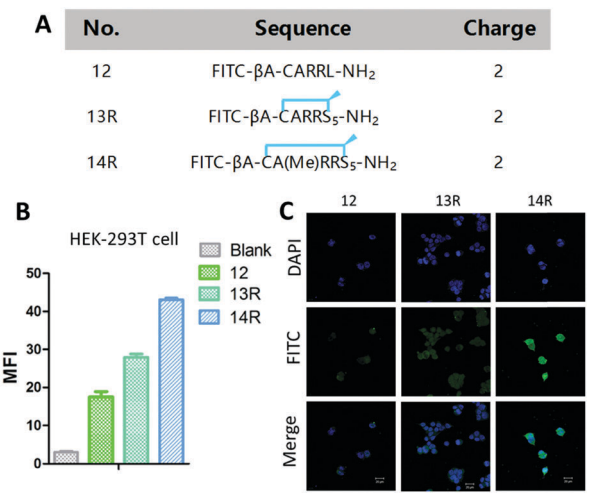

Fig. 4 N-terminus NH-methylation increases peptides cellular uptake (A) sequences and charges of designed peptides **12**, **13*R*** and **14*R***. (B) Flow cytometry analysis of the cellular uptake of peptides **12**, **13*R*** and **14*R*** in HEK-293 cells. The values represent averages of three independent experiments. (C) Confocal microscopy images of the HEK-293T cells treated with (5 μM) peptides **12**, **13*R*** and **14*R*** for 3 h at 310.15 K. Then they were washed with PBS and incubated with 0.05% trypan blue for 3–5 minutes to quench the potential peptides adsorbed at the cell surface for later confocal microscopy imaging.[15] The scale bars are 20 μm.

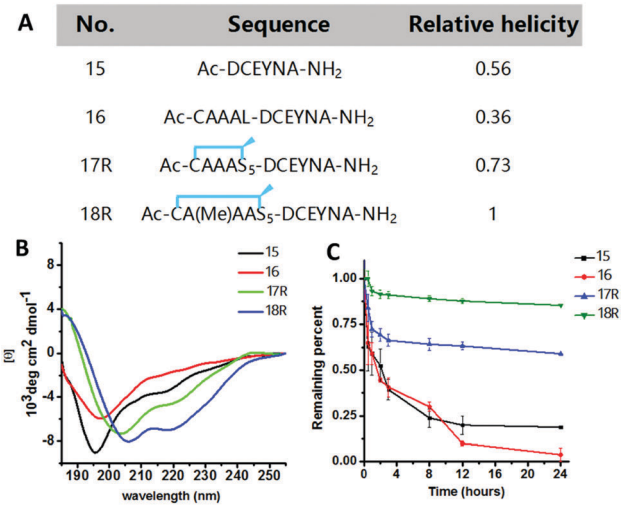

Fig. 5 N-terminus NH-methylation strategy could be utilized in longer peptides. (A) Sequences of peptides **15**, **16**, **17*R*** and **18*R***. (B) CD spectra of peptides **15**, **16**, **17*R*** and **18*R*** (100 μM) in ddH_2O at 298.15 K. (C) Serum stability assay of peptides **15**, **16**, **17*R*** and **18*R*** (250 μM) in mouse serum (25%) for 24 h at 310.15 K.

To further explore the biophysical influence of *N*-methylation on CIH peptides, Arg-containing peptides were prepared as shown in Fig. 4A. Flow cytometry analysis (FACS) of HEK-293T cells (Fig. 4B) and HeLa cells (Fig. S7, ESI†) treated with these peptides was performed. The FACS results showed a significantly improved cellular uptake of peptide **14*R*** compared with peptides **12** and **13*R*** in both HEK-293T and HeLa cells. Further confocal imaging experiments also indicated that peptide **14*R*** showed better cellular uptake (Fig. 4C). These results indicated that appropriate *N*-methylation of CIH peptides may increase the peptide's rigidity and hydrophobicity, and finally result in enhanced cellular uptake (Fig. S8, ESI†).

Then we further tested whether the *N*-methylation strategy could be translated into longer peptides.[13] Peptide **15**, targeting the HIF-1α transcription coactivator interaction was chosen as the model peptide.[11] Peptides **15**, **16**, **17*R*** and **18*R*** were prepared (Fig. 5A).[14] The CD spectra of peptides **16*R*** and **17*R*** showed clear helical characteristics which indicates that N-terminus methylation could be applied in longer peptides (Fig. 5B). The FACS experiments indicated that *N*-methylated long peptides showed the best cellular uptake. (Fig. S9, ESI†). Notably, *N*-methylated peptide **17*R*** showed better serum stability than its non-methylated analogue peptide **16*R***. (Fig. 5C).

In summary, we studied the effects of *N*-methylation for CIH peptides' secondary structures and revealed that masking two intra-helical hydrogen bonds would diminish the peptides' helicity although they are stabilized with the CIH method. In addition, we identified that the N-terminus NH, which does not participate in the intra-helical hydrogen bond network, is a suitable site for *N*-methylation to obtain helical and more cell permeable CIH peptides. We envisaged that the introduction of *N*-methylation could block the hydrogen bond between the N terminal NH and solvent, decrease the peptides' polar surface area and increase the peptides' hydrophobicity. In our previous study on the correlation between CIH peptides' cellular uptake and their helicity, we unambiguously demonstrated that the cellular uptake of peptide epimers directly correlated with their relative helicity.[16] In this study, we envisaged that the introduction of *N*-methylation at positions not taking part in the intrahelix hydrogen bonding network might maintain helical features, decrease the hydrogen bonds with solvent, decrease the peptides' polar surface area and increase the peptides' hydrophobicity. All these features may help to improve the peptides' cellular uptake.

This N-terminus *N*-methylation strategy could also be applied in modulating longer peptides' biophysical properties. Based on the best of our knowledge, this study is the first systemic elucidation of the synergetic combination of *N*-methylation and crosslinking strategies. This method would help the research community in peptide designing for multiple purposes.

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Conflicts of interest

The authors declare no competing financial interest.

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