ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2017, 53, 6728

Received 16th May 2017,
Accepted 29th May 2017

DOI: 10.1039/c7cc03799f

rsc.li/chemcomm

A precisely positioned chiral center in an *i*, *i* + 7 tether modulates the helicity of the backbone peptide†

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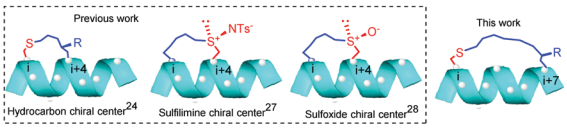
In some cases, helical peptides stabilized by an *i*, *i* + 7 tether exhibit better target binding and cellular functions compared to their *i*, *i* + 4 analogues. Herein, we carried out a systematic study of the effects of an in-tether chiral center on the *i*, *i* + 7 system. We screened the optimal cross linking mode, tether length, in-tether chiral center positions, and absolute configurations. From these studies, we determined that a chiral center of *R* absolute configuration at the γ-position to the C-terminal of a 10-membered tether could function to efficiently induce helicity of the backbone peptides. This is an important addition to the current *i*, *i* + 4 in-tether chiral center induced helicity strategy (CIH strategy), and could have broad biological applications.

Constraint peptides with preferable biophysical properties have been developed to target various protein–protein interactions (PPIs).[1–10] The side chain cross-linking strategy is a critical strategy in the development of stabilized peptides, and numerous chemical methods have been applied to generate peptides with fixed secondary structures (mostly helical) for various purposes.[11–23] We recently reported that a precisely positioned chiral centre in an *i*, *i* + 4 tether could dominate the backbone peptide's helicity, target binding affinity, and cellular uptake (chirality induced helicity strategy, **CIH**).[24,25] The validity of this **CIH** strategy was verified using both an in-tether carbon chiral center and a sulfur chiral center.[26–29] The synergetic effect was also reported in the dual in-tether chiral centers (**DCIH**) system to constrain a peptide.[30] With the exception of helicity stabilization, a sulfilimine chiral center in an *i*, *i* + 3 tether was found to induce a type-III β turn structure.[26] These results indicate that the **CIH** strategy could be applicable for the stabilization of multiple secondary structures.

The development of constraining methods between different positions is desirable. In order to choose a proper tethering position is largely dependent on both the sequence and the target. In some cases, constraint peptides that are stabilized with a two helical turn tether, namely an *i*, *i* + 7 tether, could provide better helix stabilization, target binding and cellular functions than a single turn *i*, *i* + 4 tether.[5,31–33] Compared to the single turn tether, the two turn tether generally requires more atoms (*i.e.* an 8 atom tether for an *i*, *i* + 4 all hydrocarbon stapled peptide and an 11 atom tether for an *i*, *i* + 7 stapled peptide). When taking the tether length and the size of the peptides into consideration, finding a properly positioned chiral center in an *i*, *i* + 7 tether is actually more challenging, which is due to the fact that this chiral center should exhibit a nucleating effect on a more flexible tether and a larger size peptide (Scheme 1).

Herein, we systematically investigated the effects of the chiral centre on an *i*, *i* + 7 tether as shown in Scheme 1. We chose an octapeptide as a model to study the in-tether chiral center effects on the *i*, *i* + 7 tether, as this could provide minimum strain and steric hindrance, and could avoid possible sequence perturbations. As demonstrated in our previous reports, a hydrocarbon chiral center could be translated into sulfoxide or sulfilimine chiral centers as shown in Scheme 1. Therefore, we began with sulfoxide chiral centers in order to screen for the proper chiral center position, for relatively simplified synthesis. As shown in Fig. 1A, a panel of macrocyclic peptides Ac-cyclo-$X_{(n)}$AAAAAAC-NH_2 (n = 7–10) tethered by thioether linkages consisting of different



Scheme 1 Schematic representation of an in-tether chiral center induced helical peptide *via* an *i*, *i* + 4 and *i*, *i* + 7 cross linking system. Notably, the L-amino acids were used for tethering the *i*, *i* + 4 system, while in *i*, *i* + 7 system, a D-amino acid was placed at the N-terminus and a L-amino acid was placed at the C-terminus.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7cc03799f

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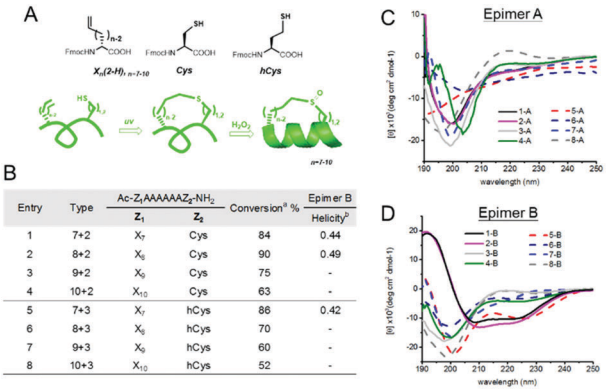

Entry	Type	Ac-Z_1AAAAAAZ_2-NH_2		Conversion[a] %	Epimer B
		Z_1	Z_2		Helicity[b]
1	7+2	X_7	Cys	84	0.44
2	8+2	X_8	Cys	90	0.49
3	9+2	X_9	Cys	75	-
4	10+2	X_{10}	Cys	63	-
5	7+3	X_7	hCys	86	0.42
6	8+3	X_8	hCys	70	-
7	9+3	X_9	hCys	60	-
8	10+3	X_{10}	hCys	52	-

Fig. 1 (A) The chemical structure of the amino acids used for constructing **ClH** peptides at i and i + 7 positions. The synthetic route of sulfoxide **ClH** peptides are shown. Abbreviation: hCys, homocysteine; Xn, (***R***)-alkenylglycine. (B) A panel of peptides used for optimizing the cross-linker length and chiral center position. Notes: [a]the conversion of the thiol–ene reaction was determined by LC-MS integration. [b]The percentage of helicity for the B epimer was calculated based on the Luo and Baldwin rule.[13] "–" indicates the helical content is unavailable. (C and D) CD spectra of the sulfoxide epimer A or B (~20 μM) of peptides **1–8**, H_2O, 20 °C, respectively.

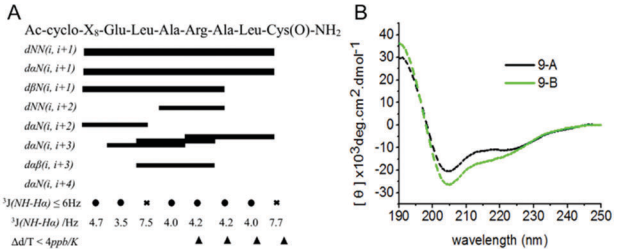

Fig. 2 (A) NOE summary diagram of **9-B** (measured in 10% D_2O in H_2O, 20 °C, 2 mM). Bar thickness reveals the intensity of the NOE signals. (B) CD spectra of peptides **9-A/B** (~20 μM) was measured in water at 20 °C.

numbers of atoms (a 9 to 13 membered tether with sulphur atoms at different positions) were prepared *via* a thiol–ene reaction.[3] We found that the cyclization efficiency of the radical reactions was influenced by the side chain length of amino acid X_n. An X_n with a shorter side chain, as in entry **1**, **2**, **5** exhibited higher cyclization conversion than entry **4**, **7**, **8** with a longer side chain (Fig. 1B). Circular dichroism (CD) spectroscopy studies clearly indicated that a simple thioether tether was not able to induce α-helicity (Fig. S1, ESI†), which is in agreement with our previous reports.[24,27,28,34,35]

Next, all peptides were subjected to 5% H_2O_2 oxidation in order to generate peptide sulfoxide epimers. These peptide sulfoxide epimers were then subsequently separated by reverse phase HPLC (rpHPLC). The epimers were designated as peptides A and B, referring to their respective HPLC retention time ($t_A < t_B$). For peptide entries **1** and **2**, the significant difference in retention time between the ***S*** and ***R*** epimers indicated the presence of structural variations within the solution. In the case of the other peptide entries, we found that the retention time difference was decreased, indicating little structural variation within the solution (see selected HPLC curve for **2-A/B** and **7-A/B** in Fig. S2, ESI†). The CD spectroscopy study of peptides **1–8(A/B)** with different tether lengths and chiral center positions (γ and δ position) were performed in H_2O and were summarized in Fig. 1C and D. All peptides exhibited random coil structures, while peptides **1-B** and **2-B**, both with a γ positioned chiral center (tether length of **9** or **10** atoms), exhibited significantly enhanced α-helical contents (Fig. 1D). Notably, we found that either increasing the tether length (peptide **3**/**4**) or switching the chiral center positions (peptide **5–8**) resulted in a largely diminished helical structure. We found that peptide **5-B** possessed comparable ellipticity at 222 nm with peptide **1-B** or **2-B**. However, the negative maximum ellipticity of **5-B** shifted to 202 nm (208 nm for a regular α-helix), along with a low $\theta_{222}/\theta_{202}$ ratio, which suggests the existence of a potential 3^{10} helicity/random coil structure.[14] Based on our previous study, we know that the N-γ position is non-essential in the i, i + 4 **ClH** system[24–28] (Scheme 1). Here, we aimed to understand whether this phenomenon holds true in the i, i + 7 system. We prepared the control peptides **S-I** and **S-2** with the chiral centre at the N terminus. Both of the peptides were separated as one peak in the HPLC chromatogram (Fig. S3, ESI†), which is the mixture of the *R*/*S* isomers. The CD spectra demonstrated that both of the peptides exist as random coils in H_2O (Fig. S4, ESI†). We found that the preparation of X_8 was more efficient than that of X_7, and the 8 + 2 tether was also found to be slightly better than the 7 + 2 tether in helix stabilization. Thus, we used the 8 + 2 tether as the standard linking pattern in further studies.

To better illustrate the secondary structure of the i, i + 7 peptide's conformation in aqueous solution, a detailed 1D and 2D ^{1}H-NMR spectroscopy study of peptide **9-B** (Ac-cyclo-X_8ELARALC(O)-NH_2) was performed in 90% H_2O:10% D_2O (V:V). Except for the residues Cys and a Leu (closed to N-termini), the other six residues had low amide coupling constants ($^3J_{NH-CH\alpha} \leq 6$), suggesting that they were part of helical structures (Fig. 2A). The observation in NOESY spectra of non-sequential medium-range $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOEs in **9-B** further suggests helical structures (Fig. S5, ESI†). In addition, the low temperature coefficient ($\Delta\delta/T < 4.5$ ppb K^{-1}) of Arg5, Ala6, Leu7 and Cys8 in **9-B** were also indicative of hydrogen bonding patterns typical of α helical structure (Fig. S6, ESI†). Additionally, the CD spectra of **9-A/B** were showed in Fig. 2B, which suggested **9-B** is a more helical structure than **9-A**. Therefore, the NOE and CD spectra of peptides **9-B** clearly showed that a precisely positioned chiral centre in an i, i + 7 peptide could induce helicity of the backbone peptide in aqueous solution.

We further examined whether the helical inducing effect of an in-tether sulfoxide chiral center in an i, i + 7 system could be transferred into a carbon chiral center as shown in Fig. 3A. The enantiomerically-pure unnatural amino acids S_8(Ph-*R*) and S_8(Ph-*S*) were prepared according to reported procedures.[36] Peptides **11-*S*/*R*** Ac-cyclo-$_{(d)}$CAAAAAAS$_8$(2-Ph)-NH_2-*S*/*R* were synthesized. We found that the epimers exhibited both a distinguished retention time (Fig. S7, ESI†) and CD spectra, as shown in Fig. 3B. The peptide **11-*R*** exhibited an α-helical structure while the **11-*S*** epimer exhibited a random coil structure. This confirmed the modulating effect of an in-tether hydrocarbon chiral center in

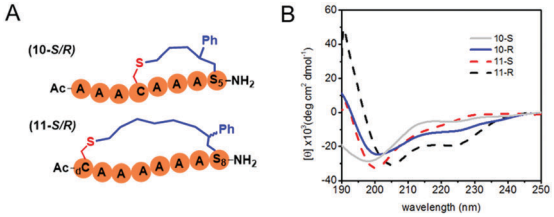

Fig. 3 (A) The chemical structures and (B) CD spectra of **10-*S*/*R***, **11-*S*/*R***. The CD measurements were performed in H_2O, 20 °C at ~20 μM. $_{(d)}$C indicates the D-cysteine.

the *i*, *i* + 7 system. The *i*, *i* + 4 tethered version of peptide **11** was also synthesized, namely peptide **10-*S*/*R***. Peptide **11-*R*** displayed obvious better helical conformation than **10-*R***. We also synthesized peptide **S-3** to demonstrate that the N-γ position is nonessential (Fig. S8, ESI†). Finally, we investigated the thermal and chemical stability of peptide **11-*R*** (Fig. S9, ESI†). The slight decrease of ellipticity observed at 222 nm by CD measurements suggests the high stability of peptide **11-*R***.

Based on these results, we enriched the chemical toolbox of constructing *i*, *i* + 7 **CIH** peptides. Several co-crystal structures of peptide modulators-MDM2/MDMX revealed that the tether is involved in interactions with the flat hydrophobic region surrounding the target ligand binding site. In addition, it was shown that a larger hydrophobic tether of the *i*, *i* + 7 stapled peptide exhibited enhanced performance over their *i*, *i* + 4 analogues.[31–33] Thus, the development of a **CIH** peptide modulator of p53-MDM2/MDMX interactions (PDI[36,37]) would provide a suitable model for the study of performance differences between *i*, *i* + 4 and *i*, *i* + 7 **CIH** peptides. Peptides **13**-FITC-βA-LTF[cyclo-CEYWS$_5$(Ph-*R*)]QLTSAA-NH$_2$, **14**-FITC-βA-LTF[cyclo-$_{(d)}$CEYWAQLS$_8$(Ph-*R*)]SAA-NH$_2$, and their linear peptide **12** were prepared as shown in Fig. 4A. We showed that the *i*, *i* + 7 tethered peptide exhibited enhanced helical content compared to the *i*, *i* + 4 tether (19.6% and 15.7%, respectively, Fig. 4B), and both of them were more helical than peptide **12**. As shown in Fig. 4C, fluorescence polarization assay demonstrates that the *i*, *i* + 7 **CIH** peptide exhibited enhanced binding affinity with MDM2 protein (374 nm). This phenomenon could be attributed to the higher helical content of *i*, *i* + 7 peptide, as well as its enlarged tether interaction interface, observations that are in agreement with previous reports.[38,39]

In addition, *in vitro* serum stability assays showed that the *i*, *i* + 7 peptide exhibited increased resistance to protease degradation and remained approximately 85% intact after 24 hours (Fig. 5A). We also evaluated cellular uptakes of the *i*, *i* + 7 peptide or *i*, *i* + 4 peptide by fluorescence-activated cell sorting (FACS). These studies showed that the *i*, *i* + 7 peptide possessed higher cellular uptake compared to the *i*, *i* + 4 peptide (Fig. 5B and Fig. S10, ESI†). These results were further confirmed by fluorescence confocal imaging (Fig. 5C). Thus, the *i*, *i* + 7 **CIH** peptide was determined to have better biophysical characteristics than its *i*, *i* + 4 **CIH** analogue in the p53/MDM2 model study. This observation is in agreement with previous reports of stapled peptides.[31,39,40]

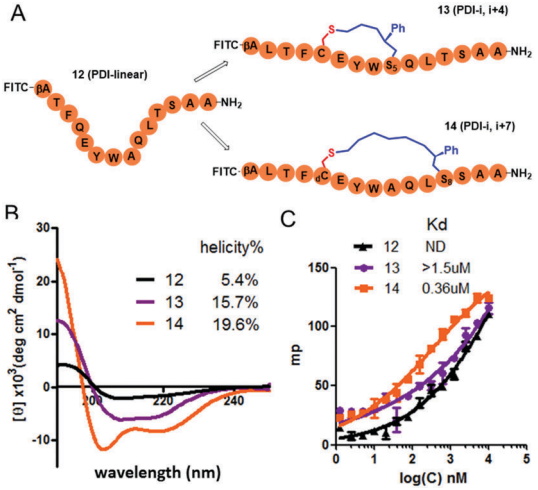

Fig. 4 (A) Representation of **12**, **13** (PDI-*i*, *i* + 4) and **14** (PDI-*i*, *i* + 7 **CIH**) peptide chemical structures. (B) The comparison of mean residue helicity of **12**, **13** (*i*, *i* + 4) and **14** (*i*, *i* + 7) **CIH** peptides. CD spectral measurements were performed in H_2O, pH 7, 20 °C. (C) Binding affinity of **12**, **13** (PDI-*i*, *i* + 4) and **14** (PDI-*i*, *i* + 7) with MDM2, respectively. The binding affinities were measured using fluorescence polarization assays (FPA) at 20 °C.

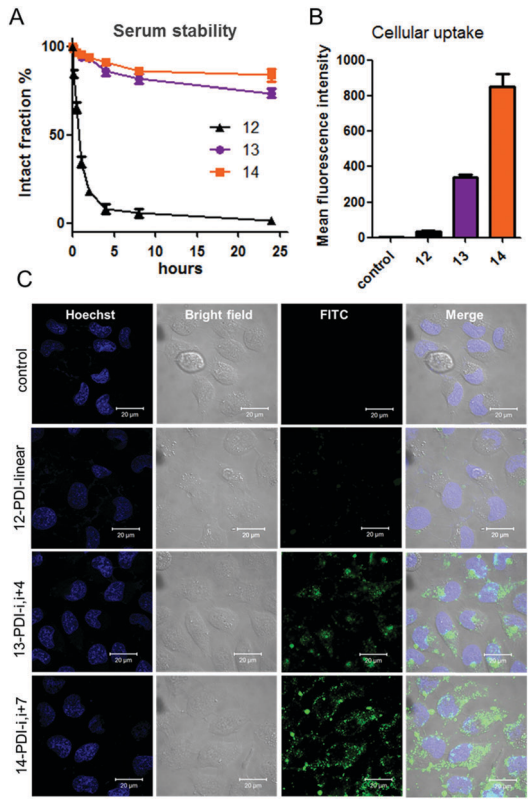

Fig. 5 (A) *In vitro* serum stability of **CIH** peptides. Peptides **12–14** were incubated with human serum at 37 °C. (B) Comparison of cellular uptakes of peptides **12**, **13** and **14**. Hela cells were incubated with peptides (5 μM) for 2 hours and the fluorescence intensity was measured by flow cytometry. Error bars represent the standard error of mean (SEM) from more than two experiments. (C) Live cell confocal images of peptides **12**, **13** and **14** (5 μM, 37 °C) Hela cells treated for 1 hour. Hoechst 33258: blue; FITC: green.

In this report, we systematically studied the *i*, *i* + 7 **CIH** system, from an in-tether sulfoxide chiral centre to an in-tether hydrocarbon chiral centre. Both were found to demonstrate positive modulating effects on the helicity of the backbone peptides. The p53/MDM2 modulator model study revealed that the *i*, *i* + 7 **CIH** peptide exhibited superior biophysical properties compared to its *i*, *i* + 4 analogue. This suggests that this method could be used for a range of biological applications. Such studies will enable a better understanding of the origin of peptide secondary structures, as well as enrich the chemical space of conformationally constrained peptides.

This work is supported by the Natural Science Foundation of China Grants 21372023 and 81572198; MOST 2015DFA31590, the Shenzhen Science and Technology Innovation Committee JSGG20140519105550503, JCYJ20150331100849958, JCYJ20150403101146313, JCYJ20160301111338144, JCYJ20160331115853521 and JSGG20160301095829250; the Shenzhen Peacock Program KQTD201103.

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