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COMMUNICATION

Switching Substitution Groups on the In-tether Chiral Centre Influences the Backbone Peptide' Permeability and Target Binding Affinity

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Different substitution groups on the in-tether chiral centre of the chirality-induced helical peptides (CIH peptides) showed distinguishable effects on peptides' cellular uptakes and binding affinities with estrogen receptor α(ER-α). This study proves that in-tether chiral centre is a valuable modification site for constructing peptide ligands with preferable biophysical properties.

Protein-protein interactions (PPIs) play pivotal roles in mediating intracellular biological processes and targeting dysfunctional PPIs is broadly utilized for therapeutics development.[1] However, small molecule ligands are less likely to interrupt PPIs with large, shallow or discontinued surfaces, which makes many PPIs "undruggable".[2] Over 50% PPIs involve α-helices interactions, however; although peptides could efficiently interrupt PPIs *in vitro*, they suffer from poor stability and cell permeability. In past decade or so, constraint helical peptides stabilized by various chemical means with enhance druggability were intensively studied to construct suitable peptide-ligands for various PPIs.[3]

Recently, Moore *et al.* reported that a chiral centre on the tether of a stapled peptide could affect the peptide's secondary structure and binding affinity. Meanwhile, we reported that a precisely positioned carbon chiral centre in a single bonded tether was capable of modulating the helicity, cell permeability and binding affinity of a peptide.[4] Both groups clearly stated that the substitution group on the in-tether chiral centre could be of important modulating effects on backbone peptides' biophysical properties, without any alteration on the backbone peptides (Fig. 1A).

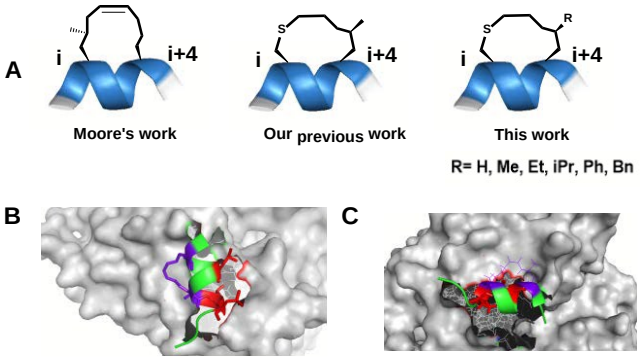

Fig. 1 (A)Stabilized peptides with an in-tether chiral centre developed by *Moore et al.* and us. (B) Crystal structure of stapled peptide bound to estrogen receptor.

Although conceptually the artificial tethers were designed to point at the solvent face to avoid direct interactions with target protein; however, in some reported cases, the tether counted significantly for the overall ligand-target interaction, such as ER-α and MDM-2. *Phillips et al.* reported an example of replacing interacting residues with a hydrocarbon staple. The crystal structure of stapled peptide PFE-SP2 bound to estrogen receptor α (ERα) showed that an i, i + 4 hydrocarbon staple can replace isoleucine and leucine residues on the binding face of a steroid receptor coactivator 2 (SRC2) peptide (Fig. 1B). They found that this change receives an increase in helical content and binding affinity. SRC2 interacts with the surface of ERa over two turns of an a-helix using an LXXLL motif (X is any amino acid).[5] Moore et al. also reported the complex structure of the chiral center bearing stapled peptide with ERα in a similar pattern (Fig. 1C).[4]. In Moore's and our previous reports, both of us found the in-tether chiral centre showed significant influences on the peptides' binding affinity with ER-α.[4] However, how the substitution groups on the in-tether chiral centre influence the backbone peptides' biophysical properties still lacks systematic study.

In this report, a panel of **CIH** peptides targeting ER-α with identical peptide backbone but different branches at the in-tether chiral centre were constructed as shown in Fig. 2A. Six

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Electronic Supplementary Information (ESI) available: experimental procedures, supporting tables and figures. See DOI: 10.1039/x0xx00000x

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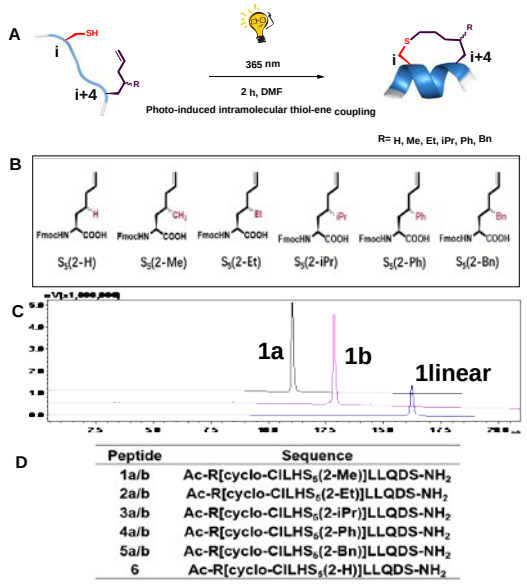
unnatural amino acids with different branches utilized in this research were shown in Fig. 2B.[6] Since Moore *et al.* reported in their study that the stapled peptide without a chiral centre showed better helical content but minimal binding with ER-α comparing with stapled peptides containing a chiral centre, therefore, we prepared peptide **6**, which contains no in-tether chiral centre as a control.



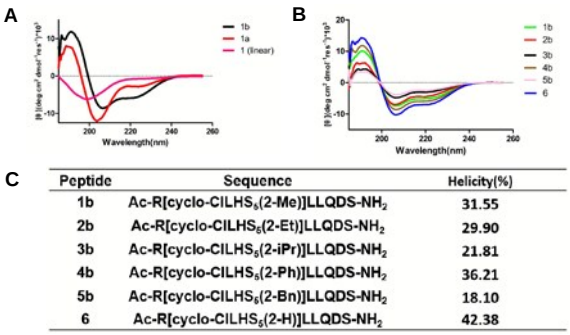
Peptide	Sequence
1a/b	Ac-R[cyclo-ClLHS$_5$(2-Me)]LLQDS-NH$_2$
2a/b	Ac-R[cyclo-ClLHS$_5$(2-Et)]LLQDS-NH$_2$
3a/b	Ac-R[cyclo-ClLHS$_5$(2-iPr)]LLQDS-NH$_2$
4a/b	Ac-R[cyclo-ClLHS$_5$(2-Ph)]LLQDS-NH$_2$
5a/b	Ac-R[cyclo-ClLHS$_5$(2-Bn)]LLQDS-NH$_2$
6	Ac-R[cyclo-ClLHS$_5$(2-H)]LLQDS-NH$_2$

Fig. 2 (A) Schematic presentation of **CIH** peptide preparation. (B) Structures of unnatural amino acids used in this study. S_5(2-Me) means there is a methyl group at the γ-position to the *S* amino acid. (C) HPLC traces of epimers **1a, 1b** and **1linear**. (D) Peptide sequences.

Peptides with different substitution groups were synthesized and the epimers were readily separated by reverse-phase HPLC. The retention time differences clearly indicated significant structural differences in solution as shown in Fig. 2C. Circular dichroism(CD) spectroscopy analysis was performed on linear peptide **1**, and two epimers **1a** and **1b** obtained by cyclization of **1**. Peptide **1b** showed significantly enhanced helical contents than its epimer **1a** while the linear peptide **1** was mainly random coil. (Fig. 3A) The CD spectra of all **b** epimers were summarized in Fig. 3B and the α-helical content of each b epimer was calculated as previous reports[7] (Fig. 3C). CD spectra of **a** epimers were summarized in Supporting Information Fig. S1 and Table S1. Notably, we also detected peptide **6** exhibited better helical content comparing with other **CIH** peptides, which was in agreement with Moore's report. [4] Remarkably, it is the only case identified in our laboratory that a control cyclic peptide showed better helical enhancement than **CIH** peptides.

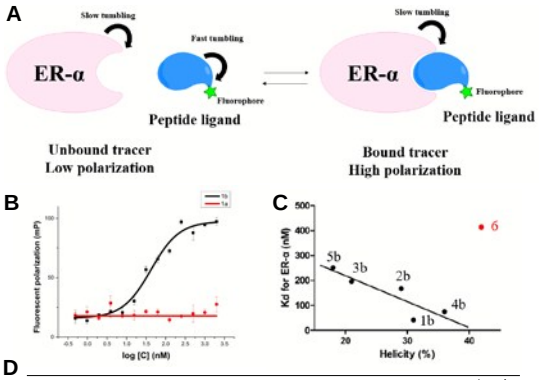
From peptide **1b**, **2b** to **3b**, the helicity declined gradually, which may suggest branched substitution groups are not preferred. Similar result was observed with peptide **5b,** which was even less helical. Notably, peptide **4b** was observed to be more helical and the rigid planar shape of phenyl may account for the enhancement.

The fluorescein-labelled **CIH** peptides FITC-**1a/1b** to **6** were tested for their binding affinity with ER-α by fluorescence polarization (FP) assay (Fig. 4A). Peptide **b** epimers showed significantly better binding with ER-α comparing with their **a** counterparts and selected examples were shown in Fig. 4B. Binding curves of other peptides were summarized in Fig. S2 and Table S2. The binding affinity of peptide FITC-**1b** to **6** was summarized in Fig. 4D. From FITC-**1b** to FITC-**5b**, the binding affinity correlated well with the peptide's helicity as shown in Fig. 4C, which clearly indicated that helicity is an important factor for **CIH** peptides' binding affinity. However, as an outliner, peptide **6** showed both the best helicity and the poorest binding affinity (Fig. 4C/4D). This observation clearly demonstrated that the substitution groups may contribute significantly for the peptide/target interaction in addition to maintaining the helical structure, which is in agreement with Moore *et al.*'s previous report.[4] Peptide **1b** exhibited the best binding affinity, which may be attributed to appropriate size and hydrophobicity of the substitution group.



Peptide	Sequence	Helicity(%)
1b	Ac-R[cyclo-ClLHS$_5$(2-Me)]LLQDS-NH$_2$	31.55
2b	Ac-R[cyclo-ClLHS$_5$(2-Et)]LLQDS-NH$_2$	29.90
3b	Ac-R[cyclo-ClLHS$_5$(2-iPr)]LLQDS-NH$_2$	21.81
4b	Ac-R[cyclo-ClLHS$_5$(2-Ph)]LLQDS-NH$_2$	36.21
5b	Ac-R[cyclo-ClLHS$_5$(2-Bn)]LLQDS-NH$_2$	18.10
6	Ac-R[cyclo-ClLHS$_5$(2-H)]LLQDS-NH$_2$	42.38

Fig. 3 (A) CD spectra of linear peptide **1** and **1a/1b** in 20% TFE 10 mM phosphate buffer solution, pH=7.4, at 20°C. (B) CD spectra of **b** epimers of all different chiral centres and H atom as a control in 20% TFE 10 mM phosphate buffer solution, pH=7.4, at 20°C. (C) Percentage of helicity of all **b** epimers, each peptide was calculated as previous report[7].



Peptide	Sequence	Kd for ER-α (nM)
FITC-1b	FITC-βA-R[cyclo-ClLHS$_5$(2-Me)]LLQDS-NH$_2$	42
FITC-2b	FITC-βA-R[cyclo-ClLHS$_5$(2-Et)]LLQDS-NH$_2$	167
FITC-3b	FITC-βA-R[cyclo-ClLHS$_5$(2-iPr)]LLQDS-NH$_2$	196
FITC-4b	FITC-βA-R[cyclo-ClLHS$_5$(2-Ph)]LLQDS-NH$_2$	75
FITC-5b	FITC-βA-R[cyclo-ClLHS$_5$(2-Bn)]LLQDS-NH$_2$	252
FITC-6	FITC-βA-R[cyclo-ClLHS$_5$(2-H)]LLQDS-NH$_2$	415

Fig. 4 (A) Schematic presentation of fluorescence polarization (FP) assay. (B) Binding of 10 nM peptide **FITC-1a/b** with ER-α at 20°C. Buffer system, 10μM 17-β-estradiol, 20mM Tris-HCl pH 8.0, 25mM NaCl, 10% glycerol and 1mM TCEP. mP, mean ± s. d. and n = 3. Non-linear regression analysis by Origin 8.0 (C)

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Relationship between peptides' helicity and binding affinity. (D) Binding affinity of peptide **FITC-1b-5b** and **FITC-6.**

The peptides' cellular uptakes and stability were then tested. Flow cytometry analysis was utilized to quantify the cellular uptakes of peptides in Hela cells. The results clearly indicated that peptide **b** epimers showed better cellular uptakes than their **a** counterparts, with a 1.2-2.5 folds increment as shown in Fig. 5A. Confocal microscopy imaging showed peptide **b** epimers distributed diffusely into cell and the majority of peptides was localized in the cytoplasm. Notably, a significant fraction of peptide **4b** was also detected in the nucleus (Fig. 5D and Fig. S3). For peptide **1-5b**, their permeability correlated relatively well with their helical contents as shown in Fig. 5B. Again, peptide **6** is an outliner as shown in Fig. 5A and B. The relatively poor cellular uptake of peptide **6** may be explained by the tether hydrophobicity difference. However, a conclusive elucidation of why peptide **6**'s high helical contents didn't improve its target binding affinity and cellular uptake is still absent and more persuasive assays may need be developed for explanation.

The cyclic peptides' stability was significantly enhanced comparing with their linear analogue. Per the in vitro serum stability assay shown in Fig. 5C, linear peptide precursor of peptide **6, 6-linear** degraded in a few hours, while peptides **1b**-**5b** and **6** remained more than 60 percent intact after 24 hours.

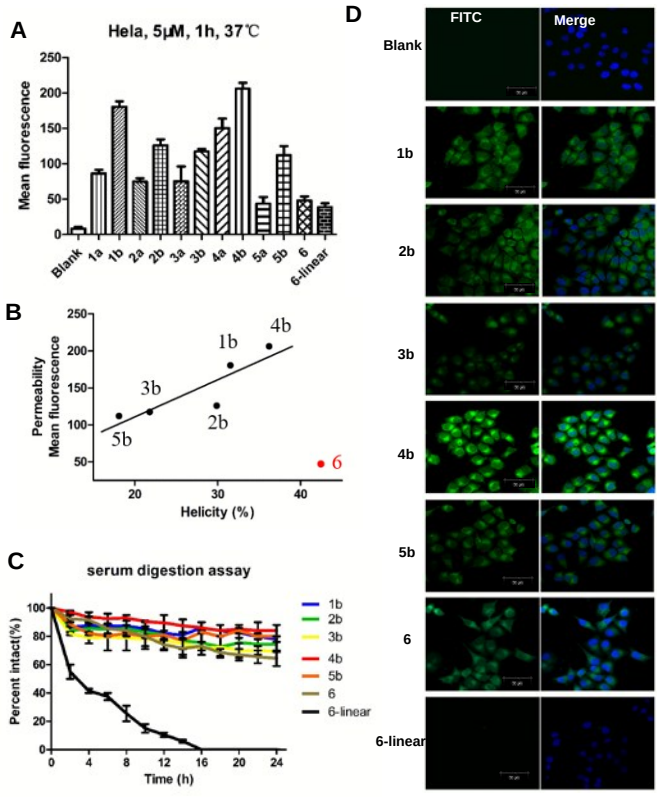

Fig. 5 Permeability and stability of peptides. (A) Flow cytometry measurements of Hela cells with 5 μM FITC-labelled peptides at 37 ℃ for 2h. (B) Relationship between peptides' helicity and permeability. (C) Serum stability measurements with 100 μM FITC-labelled peptides at 37 ℃ for 0–24h. Percentage intact, mean ± s. d. and n = 3. (D) Fluorescent confocal microscopy images of Hela cells treated with 5 μM FITC-labelled peptides at 37 ℃ for 4h (DNA, blue (DAPI) peptides, green (FITC)).

As a conclusion, the substitution groups on the in-tether chiral centre of **CIH** peptides showed significant influences on backbone peptides' helical contents, target binding affinity and cellular uptakes. This proof-of-concept study unambiguously showed that the **CIH** strategy provides a valuable in-tether modification site without alterations in the backbone peptides. This modification site could be utilized for various modifications, biological applications and SAR studies of peptide therapeutics. In addition, the **CIH** strategy provides a unique way to study the function differences casted by soly conformational differences between peptide epimers. Further investigation of the in-tether modification site is undergoing in the laboratory and will be reported in the due course.

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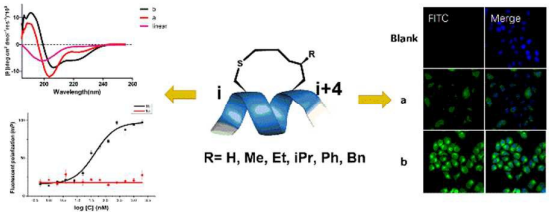

Different In-tether chiral centre show distinguishable properties proving In-tether chiral centres are valuable modification site for constructing peptide ligands.

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