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Communication

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Investigation of Cellular Uptakes of the In-Tether Chiral-Center-Induced Helical Pentapeptides

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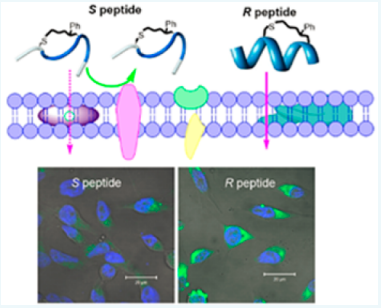
Supporting Information

ABSTRACT: We recently reported that a precisely positioned in-tether chiral center can modulate backbone peptides' secondary structures, which provides an unbiased platform to evaluate peptides' biophysical properties solely imposed by secondary structure differences. In this work, we studied the cellular uptake efficiency and mechanism of epimer pairs of a panel of chirality-induced helical peptides (CIH peptides). Although the peptides' cellular uptake is a synergetic result of various factors, our results unambiguously indicate that helical content is an important factor for the cellular uptake of CIH peptides.



Cell membranes are dynamic three-dimensional structures that protect the cell from damage. However, they are also the main obstacle for the uptake of therapeutics, especially large biomolecules like peptides and proteins. Determining the factors that affect the cellular uptake of peptides and developing methods to improve their cell permeability is of great interest to both academia and industry.[1−5] Many studies have shown that secondary structure plays an important role in the cell permeability of peptides.[6−11] Walensky and co-workers reported that helical content is an important fatctor in designing cell-permeable stapled peptides.[12] Others have also indicated that secondary structure affects the peptide uptake.[13−16] However, the relationship between the secondary structure of peptides and their cell permeability is still elusive and lacks direct evidence. Linear peptides can adopt diverse secondary structures when they interact with the lipid bilayer. More importantly, the investigation and evaluation of cellular uptake of peptides lack proper controls. Comparing the different chemical compositions of peptides can be misleading because of the unpredictable changes in biophysical properties caused by amino acid mutations and/or scrambling. We recently reported that a precisely positioned in-tether chiral center can induce helicity in peptides (the CIH strategy). Peptide construction using the CIH strategy produces two peptide epimers that are identical in chemical composition but significantly different in secondary structure.[17,18] In this study, we used peptides constructed via the CIH strategy to study the correlation between the secondary structure of peptides and their cellular uptake. The helical peptide epimers (*R* epimers) showed significantly better cell permeability than the corresponding *S* epimers (Figure 1).

The cellular uptake of peptides is synergistically regulated by multiple factors, including hydrophobicity, charge, and secondary structure. To exclude any possible perturbation from sequence variations, we chose pentapeptides as a simplified model. A series of fluorescein isothiocyanate (FITC)-labeled pentapeptides were synthesized. Since the positive charge of Arg or Lys can significantly influence the cellular internalization of peptides,[19,20] we designed peptides with variable charges. Peptides 1−4 were neutral, and peptides 5−12 had different positive charges (Figure 2A).

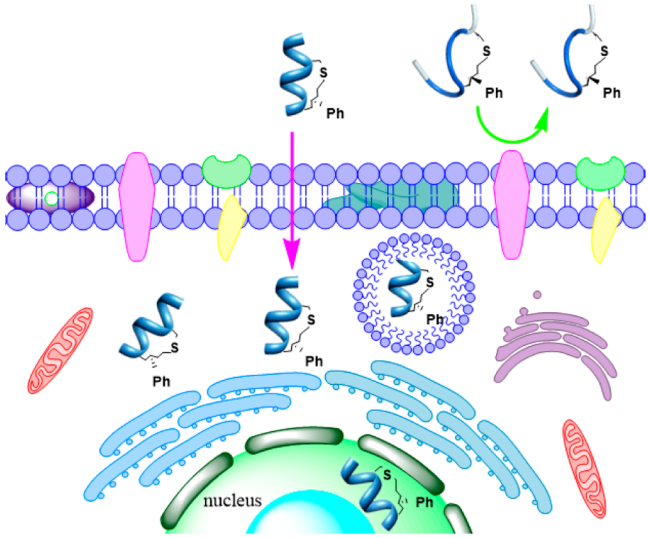


Figure 1. Overview of the correlation between peptide conformation and permeability in CIH peptides.

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No.	Entry	Sequence	Charge	S isomer helicity	R isomer helicity
1	AAA(Me)	FITC-βA-CAAAS$_5$(2-Me)-NH$_2$	0	N/A	0.87
2	AAA(Ph)	FITC-βA-CAAAS$_5$(2-Ph)-NH$_2$	0	N/A	1
3	AGA(Ph)	FITC-βA-CAGAS$_5$(2-Ph)-NH$_2$	0	N/A	0.61
4	AIA(Ph)	FITC-βA-CAIAS$_5$(2-Ph)-NH$_2$	0	N/A	0.42
5	AKA(Ph)	FITC-βA-CAKAS$_5$(2-Ph)-NH$_2$	1	N/A	0.46
6	ARA(Ph)	FITC-βA-CARAS$_5$(2-Ph)-NH$_2$	1	N/A	0.38
7	ARR(Me)	FITC-βA-CRRAS$_5$(2-Me)-NH$_2$	2	N/A	0.29
8	ARR(Ph)	FITC-βA-CRRAS$_5$(2-Ph)-NH$_2$	2	N/A	0.31
9	RAR(Ph)	FITC-βA-CRARS$_5$(2-Ph)-NH$_2$	2	N/A	0.32
10	RER(Ph)	FITC-βA-CRERS$_5$(2-Ph)-NH$_2$	1	N/A	0.28
11	RRR(Ph)	FITC-βA-CRRRS$_5$(2-Ph)-NH$_2$	3	N/A	0.16
12	rrr(Ph)*	FITC-βA-crrrD$_5$(2-Ph)-NH$_2$	3	0.16	N/A

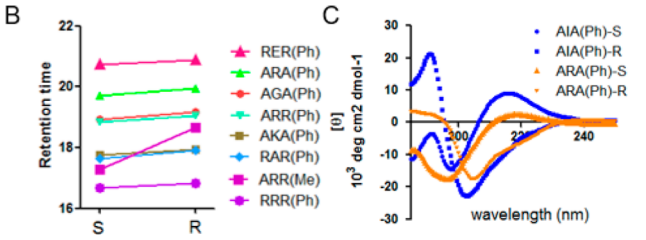

Figure 2. (A) Sequences, charge numbers, and percent helicity contents of peptides in 1× PBS (pH 7.0) at 20 °C. The *S* epimers showed a random coil structure with positive θ_{222} values based on their CD spectra, and thus, their helical content is denoted as N/A. The α-helical content of each *R* peptide epimer was calculated using the reported formula,[21] with the helicity of peptide AAA(Ph)-*R* fixed at 100%. *The rrr(Ph)-*S* epimer showed left-hand helicity in the CD spectrum, while the rrr(Ph)-*R* epimer showed a random coil structure. (B) Comparison of HPLC retention times of selected peptides. (C) Circular dichroism spectra of AIA(Ph)-*S*/*R* and ARA(Ph)-*S*/*R* in H_2O. The CD spectra of peptides in other solvents are shown in Figure S1.

During reversed-phase HPLC purification of the peptide epimers, the retention time correlations were *S* epimer < *R* epimer (Figure 2B). This may be explained by the fact that the helical *R* epimers have fewer exposed hydrogen-bond donors and acceptors than the *S* peptides, a point that structurally distinguishes the two epimers.

Circular dichroism (CD) spectroscopy analysis of the epimer pairs of peptide AIA(Ph) and peptide ARA(Ph) was conducted. The *R* epimer peptides were helical in water, while the *S* epimer peptides were mainly random coils without helical tendency (Figure 2C). (CD analyses of the other sequences have been reported in previous literature.[17]) A summary of the relative helical contents of the peptides is presented in Figure 2A,[21] which clearly indicates the significant structural differences between the *S* and *R* epimers. For all of the peptides, the *R* epimer showed a higher helical content than the *S* epimer (except for peptide rrr(Ph), which was composed of D-amino acids; its *S* epimer showed higher helical content than its *R* epimer). Residues like Ala, Lys, or Ile were conducive to high helical content (entries 1, 2, 4, and 5). Notably, Gly was tolerated in the helix (entry 3).

The CD spectra of peptides AIA(Ph) and ARA(Ph) in trifluororoethanol (TFE) and MeOH showed little variation in aqueous solution Figure S1. MeOH triggers a β-sheet conformation, and TFE usually promotes an α-helix conformation by mimicking the passage across a membrane into consideration; these results indicate that the secondary structure of CIH peptides is tightly stabilized under diverse conditions, a characteristic that provides an ideal platform for evaluating the differential cellular uptake of peptides with identical chemical compositions but distinct secondary structures. As the peptide AGA(Ph) has the smallest side chain of all of the peptides in our table (Figure 2A), an ionic mobility assay was conducted for this peptide, and the results support the structure distinction[22,23] (Figure S2).

Flow cytometry analysis was used to quantify the cellular uptake of the peptides in HEK-293 cells (Figure 3A). Trypsin

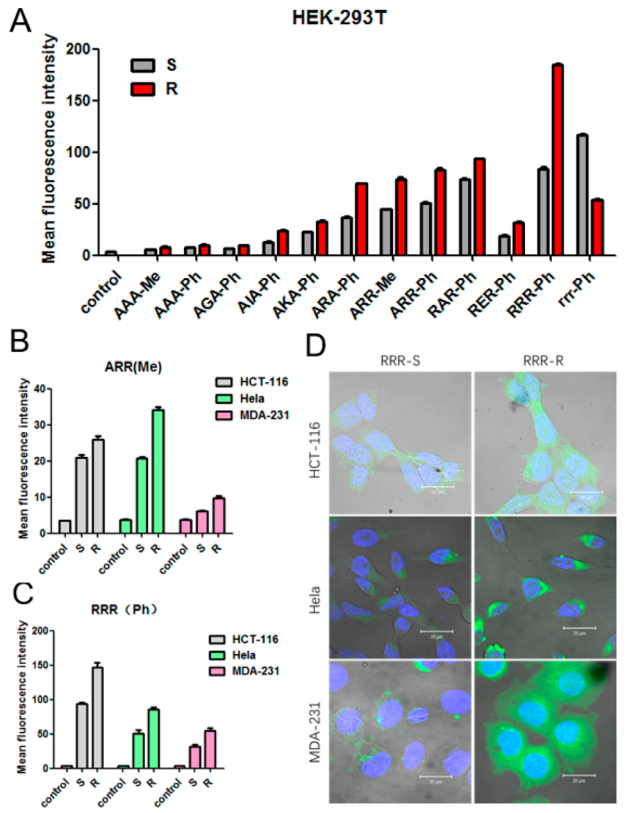

Figure 3. (A) Flow cytometry analysis of the cellular uptake of peptides in HEK-293 cells. Mean fluorescence intensity data showed a clear distinction between the cell permeabilities of *S* and *R* epimers with various sequences. Peptides (5 μM) were incubated with HEK-293 cells for 1 h at 37 °C. After washing, digestion, and resuspension in PBS, the mean intracellular fluorescence intensity was analyzed using Flowjo 7.6.1 software. The values represent averages of three independent experiments. (B, C) Cellular uptakes of peptides ARR(Me) and RRR(Ph) measured in three cancer cell lines by flow cytometry. Peptides (5 μM) were incubated with each cell line for 1 h at 37 °C and then analyzed using Flowjo 7.6.1 software. The values represent averages of three independent experiments. (D) Confocal microscopy images of the two enantiomers (*S*/*R*) of the RRR(Ph) peptide in different cell lines (MCF-7, HEK-293, and Hela). The cells were incubated with peptides (5 μM) at 37 °C for 1 h, washed with PBS, and fixed with formaldehyde for confocal image analysis. The cell nuclei were stained with DAPI (blue), while the peptides were labeled with FITC (green). The scale bars are 20 μm.

treatment was conducted before the analysis to eliminate cell-surface-bound peptides.[24] The fluorescence intensity statistics (Figure 3A) indicate that for both neutral peptides and cationic peptides, the *R* epimers with higher helical content showed higher uptakes than their *S* epimers (the only exception was peptide rrr(Ph), the helical *S* epimer of which showed a higher cellular uptake than the randomly coiled *R* epimer). Notably, the cellular uptake increased significantly as the charge of the peptide increased, an observation that is in agreement with previous reports.[2,20] As the cell membrane composition differs in different cell lines, three cancer cell lines from different

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organs (HCT-116 colon carcinoma cells, Hela cervical cancer cells, and MDA-231 breast cancer cells) were utilized to test the cell-line variances of peptides ARR(Ph) and RRR(Ph), as shown in Figure 3B,C. FACS measurements on Hela cells treated with other peptides are shown in Figure S3, and the trends observed are similar to those for the HEK-293T cell line. For all of the cell lines, the *R* epimers, which had with a higher helical contents, showed significantly better uptake than their *S* epimers (except for rrr(Ph), for which the helical *S* epimer showed a higher cell uptake than the randomly coiled *R* epimer).

The cellular uptakes of peptides 1−11 were subsequently assessed by confocal microscopy. The images of cells treated with peptide RRR(Ph) are shown in Figure 3D (see Figure S4 for other peptides) Cells of all three cell lines (HCT-116, Hela, and MDA-231) showed greater fluorescence when treated with *R* epimer peptides than when treated with *S* epimers. With the addition of positive charges, peptides 6−11 displayed enhanced fluorescence intensity under the same imaging conditions (see Figure S3). These results are consistent with the flow cytometry data.

Notably, while the helical epimers of the peptides showed significantly greater uptakes than the corresponding unstructured epimers (generally a >30% increase), the correlation between the helical content and cellular uptake of different peptides is relatively weak. No obvious correlations were observed for peptides with no charge or +1 charge (entries 1−4 and entries 5, 6, and 10, respectively). However, quantitative analysis of the peptides with +2 charge (entries 7−9) indicates that the cellular uptake of these peptides correlates relatively well with the helical content (Figure S5).

For peptides 7−9, which have similar charges and sequences, the helical content was the decisive factor for cellular uptake, and enhanced helical content correlates relatively well with increased cellular uptake. However, for peptides with significant sequence differences, it is difficult to attribute cellular uptake of peptides to a single factor such as helical content, as peptides 1−6, have only limited cellular uptakes even though they have higher helical contents than their more cationic analogues. We then tested for differences in the uptake of the mirror-image enantiomer peptides RRR(Ph)-*R* and rrr(Ph)-*S*. They exhibited significantly different uptakes, which suggests a complex mechanism possibly involving membrane receptors.

Flow cytometry assays and confocal microscopy of peptides RRR(Ph)-*S* and RRR(Ph)-*R* treated with various inhibitors of cellular uptake were also carried out to explore the potential mechanism of peptide *S*/*R* epimers (Figure S6). Since the cationic peptide RRR(Ph)-*R* showed high cellular uptake and obvious nuclear accumulation, we tested its efficiency in delivery of the anticancer reagent doxorubicin (Figure S7).[25−27]

In summary, we utilized the CIH strategy to investigate the correlation between the secondary structure of peptides and their cellular uptake. With the CIH strategy, perturbations from amino acid mutations and scrambling were avoided, and only influences due solely to secondary structure were examined. This is the first direct study of the correlation between the secondary structure and cellular uptake of peptides that are identical in chemical composition.

The cellular uptake of peptides is a synergetic result of various factors, including secondary structure. The CIH strategy provides an ideal strategy for studying the influence of helical content on cellular uptake of peptide epimers. Our results clearly indicate that helical content is an important factor in the cellular uptake of CIH peptide epimers. Notably, our study indicates that while the correlation between helicity and cellular uptake for peptides with different sequences is weak, helical content is the determining factor in the cellular uptake of peptide epimers. This study of the uptake of peptide enantiomers further suggests that a complex mechanism is at work in the uptake process.

In conclusion, the CIH strategy has been successfully applied to prove that the cellular uptake of peptide epimers increases as the helical content increases. This strategy could help us to better understand the factors involved in the cellular uptake of peptides and to design peptides with greater cell permeability.

■ ASSOCIATED CONTENT

Supporting Information

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Experimental details and additional data (PDF)

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Notes

The authors declare no competing financial interest.

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