https://doi.org/10.17952/37EPS.2024.P1200

Study on the Role of Helix-Stabilized Peptides in Transporting Antisense Morpholino Oligomers into Cells: Relationships among Helicity, Cellular Uptake, and Antisense Activity

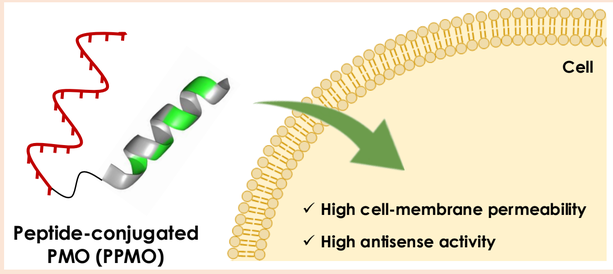
Hiroyuki Takada[1,2], Keisuke Tsuchiya[1,3], ◯Yosuke Demizu[1,2,4]

[1]National Institute of Health Sciences, [2]Yokohama City University, [3]Sanyo-Onoda University, [4]Okayama University

1. Introduction

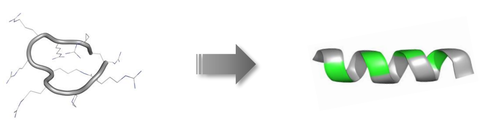
- Oligopeptides adopting well-defined secondary structures are called *peptide foldamers*. The secondary structures of cell-penetrating peptides (CPPs) influence their properties such as cell-membrane permeability, tolerability to proteases, and intracellular distribution. For example, **CPPs forming the stable α-helix structure are more cell-permeable compared with the conventional CPPs with random structures.**
- CPPs are a powerful tool for the intracellular delivery of impermeable macromolecules such as enzymes, antibodies, and oligonucleotides. In terms of the delivery of antisense oligonucleotides, several successful studies have been reported on the conjugates of cationic CPPs with phosphorodiamidate morpholino oligomers (PMOs) in cell-based assays and animal studies. However, **there are few studies where *peptide foldamers* are utilized for the delivery of PMOs.**

We designed and synthesized helix-stabilized CPPs by introducing α,α-disubstituted amino acids (dAAs) into the oligoarginine peptide and evaluated their effects on the cell permeability and antisense activity of the corresponding peptide-conjugated PMOs (PPMOs).



2. Design of helix-stabilized CPPs by introducing dAAs

Random coil → **α-helix**



Peptide	Sequence
2a	RRRRRRRRR
3a	RRX^{1}RRX^{1}RRX^{1}RRX^{1}RZ
4a	RRX^{2}RRX^{2}RRX^{2}RRX^{2}RZ
5a	RRX^{3}RRX^{3}RRX^{3}RRX^{3}RZ
6a	RRX^{4}RRX^{4}RRX^{4}RRX^{4}RZ

Z = 6-Azido-L-lysine

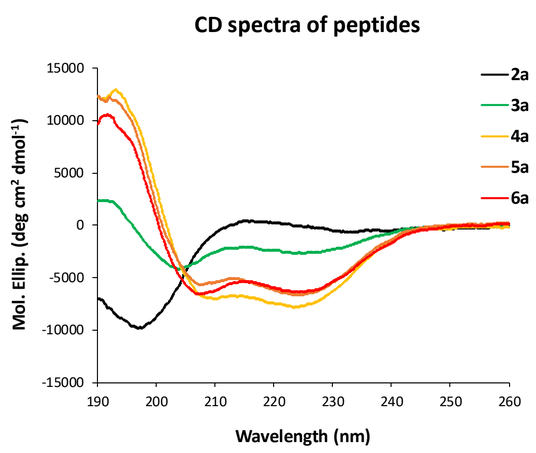
X^1 = Aib

X^2 = Ac$_4$c

X^3 = Ac$_5$c

X^4 = Ac$_6$c

CD spectra of peptides



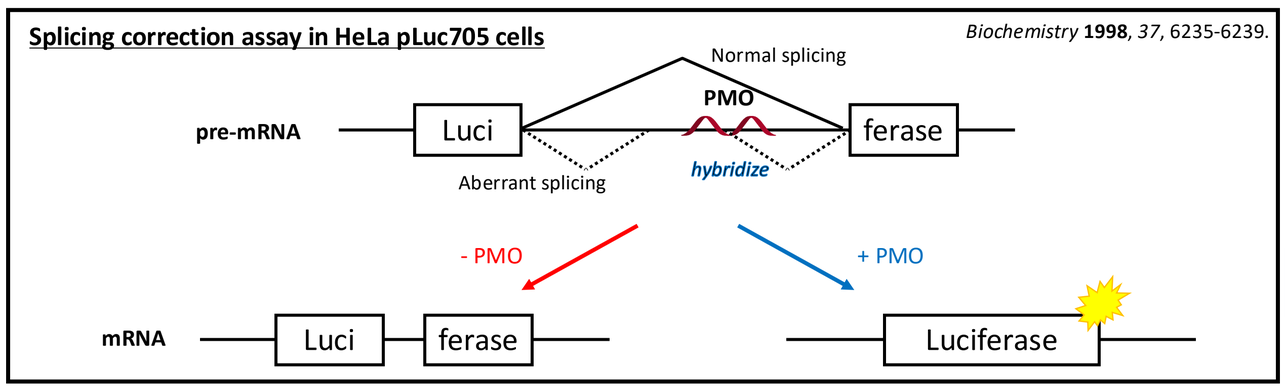
100 μM in 20 mM phosphate buffer (pH 7.4)

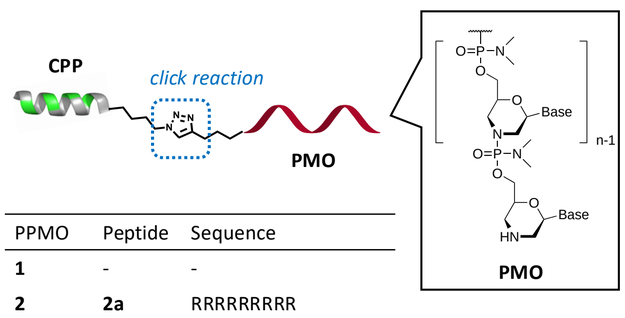
Peptides 3a–6a containing dAAs exhibited right-handed α-helix-like CD spectra, while the R9 peptide 2a showed a random-coil-like CD spectrum.

3. Antisense activity of helical CPP-PMO conjugates

Splicing correction assay in HeLa pLuc705 cells

Biochemistry **1998**, *37*, 6235-6239.

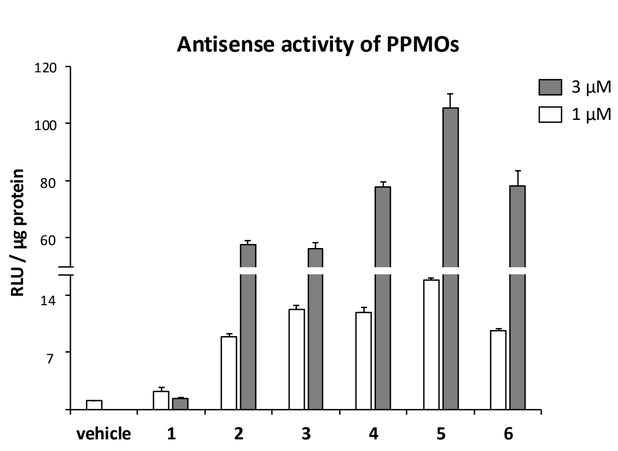




PPMO	Peptide	Sequence
1	-	-
2	**2a**	RRRRRRRRR
3	**3a**	RRX^{1}RRX^{1}RRX^{1}RRX^{1}RZ
4	**4a**	RRX^{2}RRX^{2}RRX^{2}RRX^{2}RZ
5	**5a**	RRX^{3}RRX^{3}RRX^{3}RRX^{3}RZ
6	**6a**	RRX^{4}RRX^{4}RRX^{4}RRX^{4}RZ

Z = 6-azido-L-lysine

Antisense activity of PPMOs

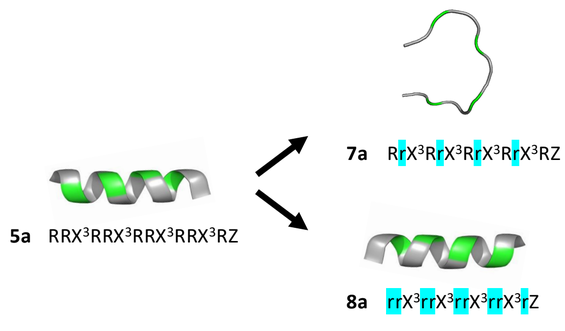


RLU: relative light unit

N = 4, Mean ± SEM

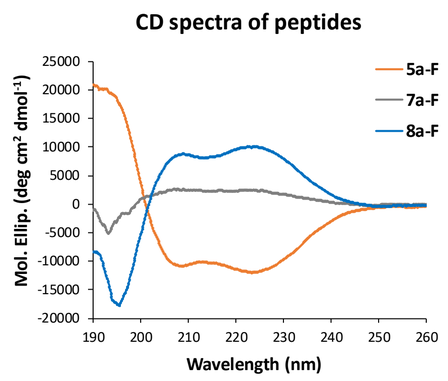
The helical CPP-PMO conjugates 3–6 exhibited higher antisense activities compared with the R9 peptide conjugate 2 in a dose-dependent manner. Among them, the conjugate 5 containing Ac$_5$c residues showed the best antisense activity.

4. Relationships among helicity, cellular uptake, and antisense activity



r = D-arginine, Z = 6-azido-L-lysine

CD spectra of peptides

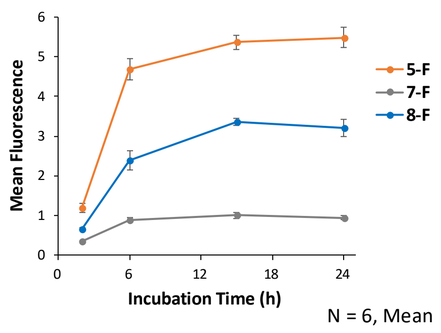


100 μM in 20 mM phosphate buffer (pH 7.4)

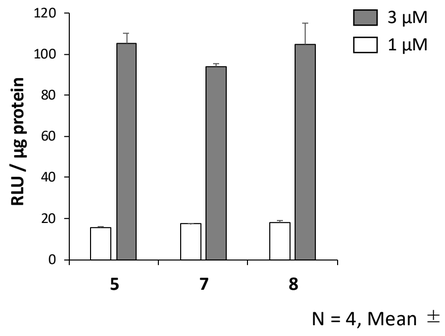
***5a-F, 7a-F, and 8a-F are modified with fluorescein at their N-terminus.**

The stereoisomers of 5a were synthesized, where the chirality of the Arg residues was altered. The peptide 7a showed a drastic decrease in helicity, while 8a exhibited a left-handed α-helical structure. These peptides have similar hydrophobicity, but different helicity.

Cellular uptake of PPMOs



Antisense activity of PPMOs



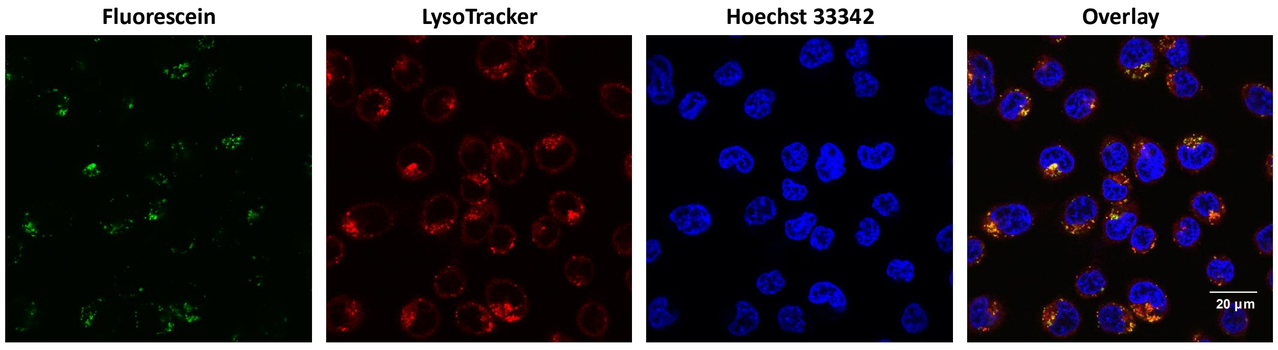
N = 6, Mean ± SEM

N = 4, Mean ± SEM

PPMO 5-F and 8-F containing helical CPPs exhibited higher cellular uptake compared with 7-F containing the non-helical CPP. By comparison, all PPMO 5, 7, and 8 showed comparable antisense activities regardless of the intracellular amounts of the PPMOs.

6. Confocal microscopy observation

Confocal microscopy images of HeLa pLuc705 cells treated with 5-F

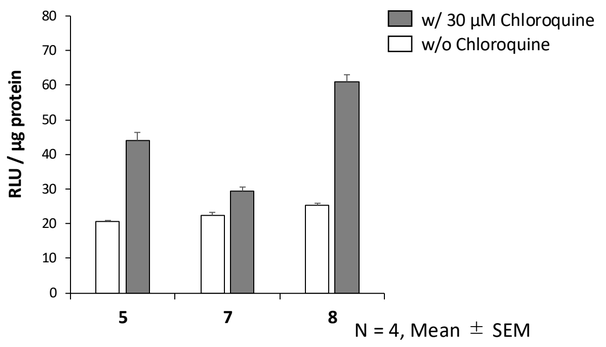


PPMOs containing arginine-rich CPPs are internalized into cells by endocytosis and they need to escape from the endosomes and move into the nuclei for the antisense activity. The confocal microscopy observation indicated that the green fluorescence of 5-F was distributed in a punctate pattern and colocalized with the endosome/lysosome marker, suggesting that most of the internalized helical CPP conjugates remained within endosomes or lysosomes and did not reach the nucleus.

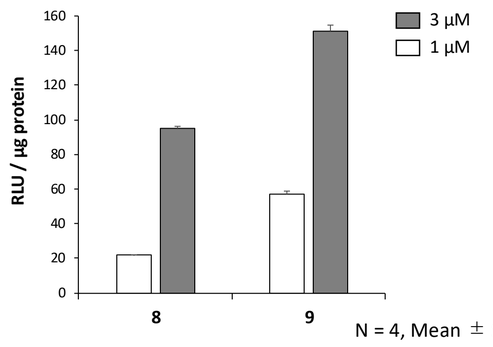
7. Synergistic effects with endosomal escape enhancers

Ahx = 6-Aminohexanoic acid

Antisense activity of PPMOs with endosomolytic reagent



Antisense activity of PPMOs with endosomal escape peptide



N = 4, Mean ± SEM

N = 4, Mean ± SEM

In the presence of chloroquine, an endosomolytic reagent, helical CPP-PMO conjugates 5 and 8 exhibited higher antisense activities than 7, suggesting synergistic effects of the efficient cellular uptake and cytosolic release of PPMOs. PPMO 9 with an endosomal escape domain peptide at its N-terminus exhibited high antisense activity compared with 8.

8. Summary

- ✓ We designed helix-stabilized arginine-rich peptides containing various dAAs to identify highly cell-permeable and potent PPMOs. Among them, the conjugate **5** containing Ac$_5$c residues exhibited a high antisense activity in the HeLa pLuc705 splicing correction assay.
- ✓ The relationships among the helicity of the peptides, cellular uptake, and antisense activity of PPMOs were investigated by using stereoisomers where the chirality of the Arg residues was altered. Consequently, it was demonstrated that helical CPPs can deliver the conjugated PMO into cells more efficiently than nonhelical CPPs, but the antisense activities were comparable regardless of the cellular uptake amounts due to endosomal entrapment.
- ✓ Co-administration of chloroquine, an endosomolytic reagent, synergistically improved the antisense activity of the helical CPP conjugates **5** and **8**, suggesting that stabilizing the helical structure of CPPs is effective to enhance the antisense activity of PPMOs when their endosomal escape is promoted.

Takada, H. *et al.*, *Bioconjugate Chemistry* **2022**, *33*, 1311-1318.