# Development of peptide-based inhibitors against β-catenin using *in silico* approach

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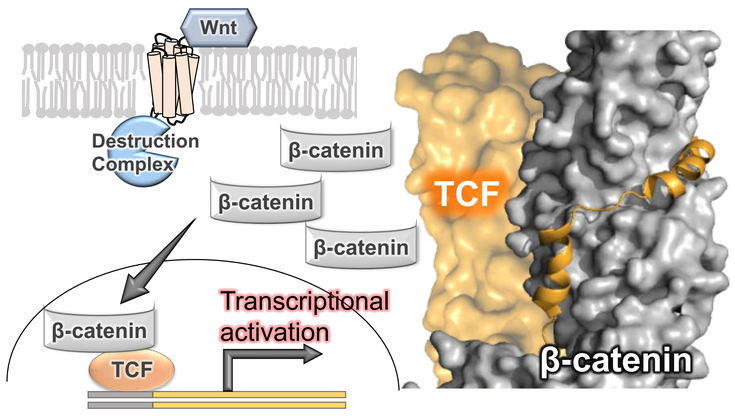
## 01 Background

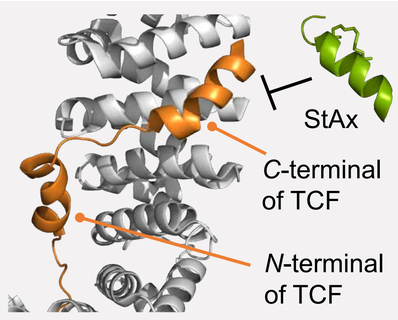
### Wnt/β-catenin signaling pathway




✧ The Wnt/β-catenin signaling pathway is transcriptionally activated through the interaction between β-catenin and T-cell factor (TCF).

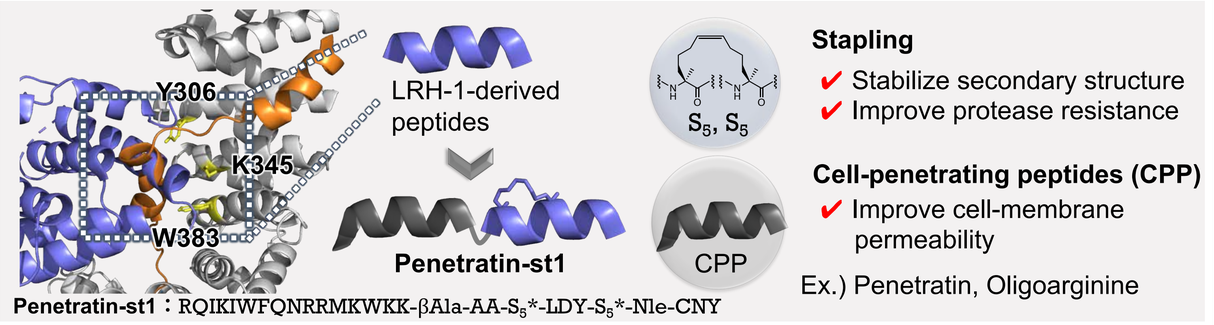
✧ Excessive transcriptional activation of Wnt/β-catenin pathway is associated with the pathogenesis of various cancers.

### Development of a peptide-based PPI inhibitor




✧ The formation of TCF is induced when TCF interacts with β-catenin. In particular, the *N*-terminal region of TCF is important for interaction with β-catenin.

✧ Axin-derived stapled peptide (StAx) that inhibits *C*-terminal helix of TCF and β-catenin showed anticancer activity.[1)]




**Stapling**
- ✔ Stabilize secondary structure
- ✔ Improve protease resistance

**Cell-penetrating peptides (CPP)**
- ✔ Improve cell-membrane permeability

1) G. L. Verdine, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 17942.
2) F. Yumoto, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 143.
3) K. Tsuchiya, *et al.*, *Bioorg. Med. Chem.* **2022**, *73*, 117021.

✧ The binding surfaces of the *N*-terminal region of TCF and LRH-1 on β-catenin partially overlap. Furthermore, the crystal structure analysis revealed that Y306, K345, and W383 of β-catenin are important for interaction with LRH-1.[2)]

✧ We designed peptide-based inhibitors using a partial sequence of LRH-1.[3)]
✧ The inhibitory activity of the **Penetratin-st1** was not sufficient (20 μM).
➢ Improving the peptide structure is required to enhance the inhibitory activity of **Penetratin-st1**.
➢ To reduce effort and costs, efficient peptide design using *in silico* methods was implemented.

## 02 *In silico* design of peptides

► An integrated scientific computing software, Molecular Operating Environment (MOE) was used to optimize the peptide sequence targeting β-catenin based on affinity scores.

### 1. Residue Scan


AALLDYTMCNYPQQT

AALLDYTMCNYPQQT
ARLLDYTMCNYPQQT
ANLLDYTMCNYPQQT
.
.
AALLDATMCNYPQQT
AALLDRTMCNYPQQT
AALLDNTMCNYPQQT
.
.

■ Each amino acid residue in the sequence was replaced by the other 19 natural amino acids.

### 2. Scoring

■ The affinity of the peptide mutants for β-catenin was evaluated by dAffinity which was measured as a difference between the binding energies of the wild-type and mutants.

Negative dAffinity
⇨Peptide-protein complex was stabilized.

Positive dAffinity
⇨Peptide-protein complex was destabilized.

| Mutation | dAffinity |
|---|---|
| C487R | -5.38 |
| D483R | -4.08 |
| C487M | -3.41 |
| . | . |

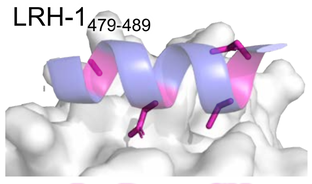
| dAffinity | Number of mutants |
|---|---|
| 0< | 114 |
| ≧0 | 146 |
| total | 260 |

Ex.) C487R
β-Catenin/peptide mutant complex was stabilized.
▷ Predicted to interact with higher affinity

■ Mutations with dAffinity value of -1 or less were extracted.

### 3. Residue Scan

■ Mutations were only introduced to amino acid residues that side chains are located at the interaction interface with β-catenin.




| Amino acid residue | Mutation |
|---|---|
| A480 | Y, F, Q, R |
| D483 | M, L, H, R |
| C487 | Y, F, L, Q, N, V, W, M, I, R |
| N488 | K |

### 4. Scoring

■ Peptides **2** and **3** which have dAffinity value of -10 or less were selected as candidates.

| dAffinity | Number of mutants |
|---|---|
| 0< | 600 |
| ≧0 | 60 |
| total | 660 |

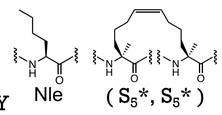
| Peptide | Sequence | dAffinity |
|---|---|---|
| **2** | ARLLLYTMRNY | -10.76 |
| **3** | AFLLLYTMRNY | -10.16 |

deficit : mutations

## 03 Synthesis of peptides

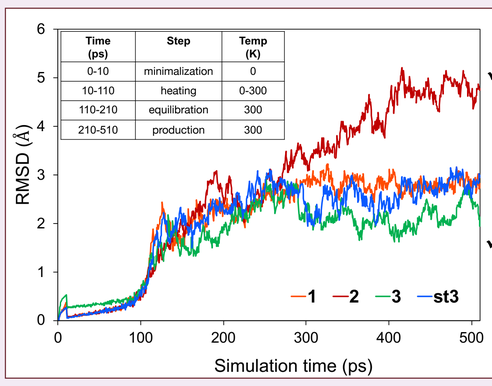
► Nle (norleucine)-derivative peptides **2** and **3** were designed. In addition, a stapled peptide **st3** and its penetratin conjugated peptide **Penetratin-st3** were also designed.

| Peptide | Sequence |
|---|---|
| **1** | βAla-AALLDYT-Nle-CNY |
| **2** | βAla-ARLLLYT-Nle-RNY |
| **3** | βAla-AFLLLYT-Nle-RNY |
| **st3** | βAla-AF-$S_5$*-LLY-$S_5$*-Nle-RNY |
| **Penetratin-st3** | RQIKIWFQNRRMKWKK-βAla-AF-$S_5$*-LLY-$S_5$*-Nle-RNY |
| **Penetratin-st1** | RQIKIWFQNRRMKWKK-βAla-AA-$S_5$*-LDY-$S_5$*-Nle-CNY |




C terminal = $NH_2$
Nle = norleucine
deficit : mutations

## 04 MD simulations



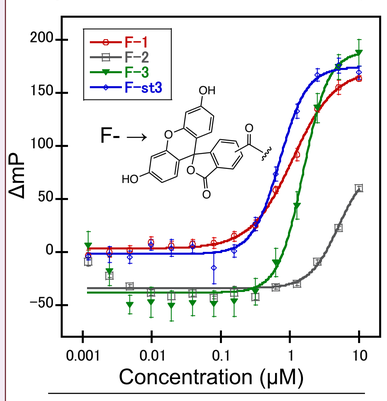

✓ Peptides **1**, **3**, and **st3** showed similar RMSD values, and were predicted to form stable complex with β-catenin.

✓ RMSD value of **2** was larger than the others.

RMSD : Root Mean Square Deviation

## 05 Binding activity

► The binding activity of 5(6)-carboxyfluorescein-labeled (F-) peptides to β-catenin was evaluated by the fluorescence polarization assay.




| Peptide | $K_d$ value (μM) |
|---|---|
| **F-1** | 1.10 |
| **F-2** | 4.99 |
| **F-3** | 1.56 |
| **F-st3** | 0.71 |

✓ Peptide **F-3**, designed using MOE, showed similar binding activity as the lead peptide **F-1**.

✓ The stapled peptide **F-st3** further enhanced binding activity.

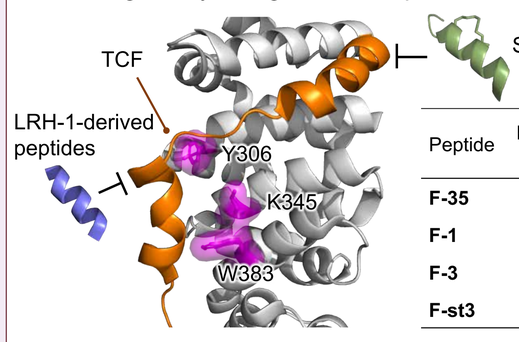
✗ Peptide **F-2**, which was expected to improve, showed reduced affinity.

MD simulation results showed that peptide **2** was more flexible than the other peptides, suggesting that peptide conformational flexibility may correlate with the binding affinity.

## 06 Interaction analysis

### 1. Mutational analysis

► The interaction surface of peptides for β-catenin was analyzed by binding assay using mutant of β-catenin (Y306A, K345A, W383A).



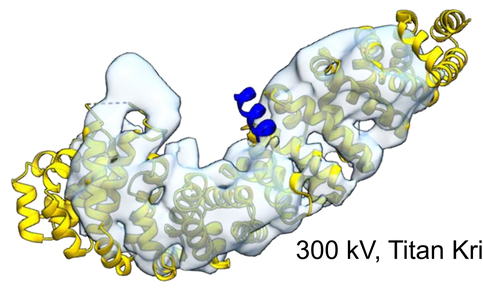

| Peptide | $K_d$ value (μM) WT | $K_d$ value (μM) MT |
|---|---|---|
| **F-35** | 0.072 | 0.054 |
| **F-1** | 0.35 | 2.1 |
| **F-3** | 0.77 | 4.2 |
| **F-st3** | 0.38 | 1.4 |

✓ The binding activity of **F-35** (StAx), which binds to β-catenin at a different region, was maintained.

✓ Peptides **F-1**, **F-3**, and **F-st3** showed reduced affinity.

### 2. Cryo Electron Microscopy

► Cryo-EM analysis of the structure of β-catenin/peptide was attempted.

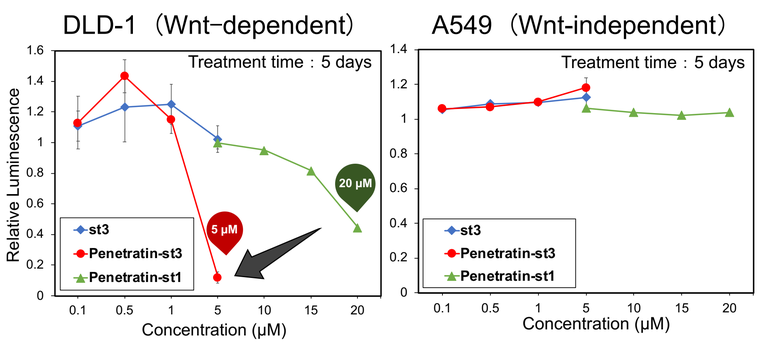


300 kV, Titan Krios G4

✓ In this experiment, a map with a resolution of 4.07 Å was obtained. Conditions are being optimized to obtain higher resolution maps to allow analysis of peptide binding sites. In addition, preparations for crystal structure analysis are underway.

## 07 Cell based assays

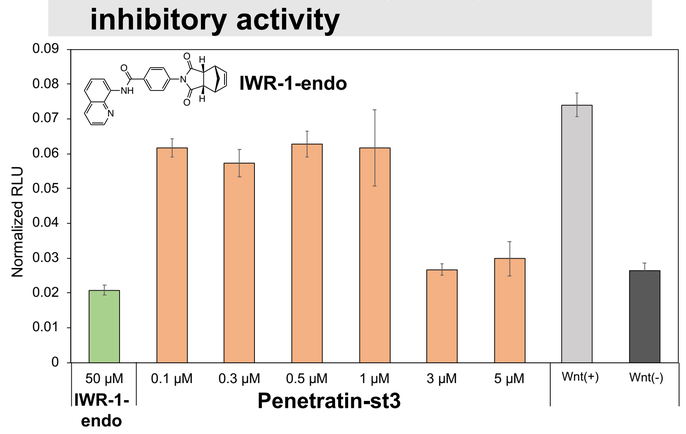
### 1. Evaluation of cell growth inhibitory activity




✓ **Penetratin-st3** inhibited the growth of only DLD-1 cells at 5 μM.
▷ The peptide has a specific growth inhibitory effect on the Wnt-signaling pathway.

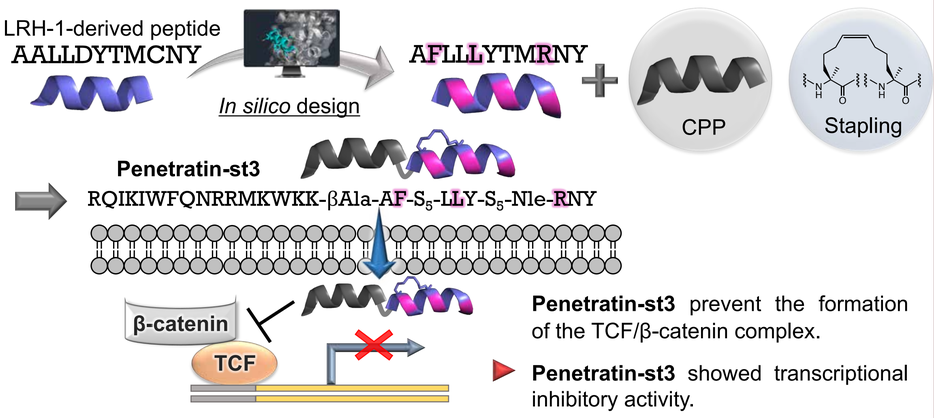
✓ **Penetratin-st3** has higher inhibitory activity than the reported peptide **Penetratin-st1**.

### 2. Evaluation of Wnt signaling-mediated inhibitory activity




✓ A concentration-dependent decrease in luminescence intensity was observed for cells treated with **Penetratin-st3**.
▷ **Penetratin-st3** intracellularly binds to β-catenin to inhibit its transcriptional activity by preventing the formation of the TCF/β-catenin complex.

## 08 Conclusion




**Penetratin-st3** prevent the formation of the TCF/β-catenin complex.

**Penetratin-st3** showed transcriptional inhibitory activity.

✓ In this study, the peptide sequence was designed using MOE.[4)]

✓ **Penetratin-st3** was found to have four-fold stronger inhibitory activity than previously reported peptides.[4)]

4) M. Fujita, *et al.*, *Bioorg. Med. Chem.* **2023**, *84*, 117264.