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Optimization of Dimeric Amphipathic Cell Penetrating Peptide

Factors Affecting the Characterization of Dimeric Amphipathic Cell Penetrating Peptide

이합체 양친매성 세포 투과 펩타이드의 최적화

길이, 아미노산의 종류, 단량체 사이 간격 면에서

2019 년 2 월

서울대학교 대학원

과학교육과 화학전공

조 제 인
Abstract

Optimization of Dimeric Amphipathic Cell Penetrating Peptide

Factors Affecting the Characterization of Dimeric Amphipathic Cell Penetrating Peptide

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Cell penetrating peptides have received much attention as a delivery tool for the cell-impermeable modulators into eukaryotic cells. However, the conventional arginine-rich CPPs need micro-molar concentrations, thus, are not suitable for delivery of the nano-molar modulators. As a remedy, we designed and synthesized bundle dimers and successfully delivered nano-molar modulators into cells. However, our first generation bundle dimeric CPP (LK-3) that has 16-amino-acid-long (16-mer) amphipathic monomers is not only cytotoxic but also difficult to synthesize for industrial use. We, therefore, designed and synthesized the second generation by adjusting three factors; length, amino acid variation and length of
spacer. As a result of adjusting the length of the monomer, we found out the optimized CPP (diLK10), in which only 10-amino-acid-long (10-mer) are used for making monomer. The cell permeability of diLK10 is lower than that of LK-3, but far better than the arginine-rich CPP (R9) and is sufficient at practical concentrations. By changing residues in hydrophilic and hydrophobic faces of the amphipathic monomer peptide, cell permeability was best when arginine and neopentylglycine (Npg) residues were in hydrophilic and hydrophobic faces, respectively. diNpgR10, diNpgK10 and diLR10 are the best when considering both the dimerization rate and the cell permeability. And next, the number of atoms between the two monomers was finely adjusted using homocysteine. When the number of atoms is 5, the cell permeability is better and the toxicity is lower than 6. For the purpose of confirming drug delivery capacity, we conjugated methotrexate (MTX) to the peptides. In MTX-resistant MDA-MB-231 cells, our short peptides increased the efficacy of MTX more than 20-fold. Especially, diNpgK10 allowed MTX to enter at a concentration as low as 40-fold, which is comparable to LK-3. In addition, all diXY10 showed lower toxicity than LK-3, especially the IC50 of diLR10 is almost 20 μM and is 4.3 times that of LK-3. Taken together, we got a new delivery tool for nano-molar modulators which can get into cells in less-toxic and more-economic manners.

**Keyword**: cell penetrating peptide, amphipathic, disulfide bond, variation, drug delivery, methotrexate

**Student Number**: 2017-20264
Contents

Abstract ................................................................................................................................. i

Contents ............................................................................................................................... iii

List of tables ......................................................................................................................... iv

List of figures ......................................................................................................................... iv

Introduction .......................................................................................................................... 1

Experimental Section ......................................................................................................... 4

1. Peptides synthesis ............................................................................................................... 4

2. Kinetic study of dimerization ............................................................................................ 6

3. Cell culture ........................................................................................................................ 7

4. Flow cytometry analysis ................................................................................................... 7

5. Cell viability assay (WST-1 assay) .................................................................................. 8

Results and Discussion ....................................................................................................... 9

1. Design, synthesis and features of peptides ........................................................................ 9

2. Adjustment of monomer peptide length ........................................................................... 17

3. Modification of residues in hydrophilic and hydrophobic face ..................................... 21

4. Adjustment of spacer length ............................................................................................. 26

5. Tendency between cell permeability and amino acids ................................................... 29

6. Biological application using CPPs ..................................................................................... 31

7. Cytotoxicity ....................................................................................................................... 33

Conclusions .......................................................................................................................... 34

References ............................................................................................................................ 36

Abstract (in Korean) ............................................................................................................. 37
List of tables

Table 1. The sequences of monomer peptides .......................................................... 12
Table 2. IC$_{50}$ of MTX in NIH-3T3, HeLa and MDA-MB-231 cell lines ........... 32

List of figures

Figure 1. The schematic structure of the dimer peptide composed of a 10-amino-acid-length monomer .......................................................... 10
Figure 2. The structures of amino acids ................................................................. 11
Figure 3. HPLC chromatograms and molecular weight of dimer peptides ....... 13
Figure 4. Dimerization rate with changes in monomer length ......................... 19
Figure 5. Cell permeability as changes in monomer length .............................. 20
Figure 6. Dimerization rate as changes of the hydrophilic and hydrophobic face 23
Figure 7. Dimerization rate of a monomer with peptide containing one Cys ..... 24
Figure 8. Cell permeability according to the type of amino acid ................. 24
Figure 9. Ratio of mean fluorescence intensity (at 62.5 nM) ......................... 25
Figure 10. The scheme of dimerization containing Hcy ..................................... 27
Figure 11. Cell permeability according to length of spacer ............................ 28
Figure 12. Cell toxicity according to length of spacer ...................................... 28
Figure 13. Hydrophobicity and cell permeability ............................................. 30
Figure 14. Comprehensive review of the changes in hydrophilicity ............... 30
Figure 15. Cell viability of MDA-MB-231 cells ................................................. 32
Figure 16. Cell viability of the second generation CPPs ................................. 33
Introduction

The cell membrane protects the inside of the cell from the extracellular environment, and selectively penetrates the necessary substances by using various proteins such as receptors, channels and pumps. However, the useful role to prevent the entry of exterior substances causes problems in drug delivery. A drug targeting intracellular substance isn’t able to exhibit the efficacy because it is merely impermeable to the cell membrane.[1] Moreover, when using a higher concentration than necessary, the risk of side effects and the cost of medicine increase. What if the drug efficacy could be delivered at a dose much lower than the current dose without developing a new drug? Several drug delivery strategies have been developed to deliver therapeutic molecules across cell membranes. One of them is a cell penetrating peptide.

Cell penetrating peptides (CPP) are peptides capable of permeating cell membranes, and are generally short peptides that consist of less than 40 amino acids. The CPP is known to the world from 1988, when the trans-activator of transcription (TAT), an HIV-1 protein, is effectively introduced into cells.[2][3] In recent years, numerous applications of CPPs have been reported that it can deliver a variety of cargo such as nucleic acids, proteins, and small molecule drugs.[4] Therefore, CPPs have received much attention as a delivery tool for cell-impermeable modulators into eukaryotic cells. CPPs have been reported to enter cells by several mechanisms, including direct pathway and endocytosis. Furthermore, in contrast to other drug delivery strategies, CPPs have many advantages such as bioavailability, ease of synthesis and modification, and low toxicity.
CPPs can be categorized into cationic class, amphipathic class, and hydrophobic class according to their physicochemical properties.[1] The cationic class is a group of multiple cationic peptides such as arginine or lysine, which include polyarginines, TAT-derived peptides and penetratin. The amphipathic class is peptides composed of both hydrophilic and hydrophobic moieties, including transportan and p28. In the case of the hydrophobic class, the major factor of cell permeability is the large affinity with the hydrophobic part of the cell membrane, and C105Y (CSIPPEVKFNKPVFVTI) and pep-7 (SDLWEMMMMVSLACQY) belong. The most well-known CPPs are TAT and polyarginines, however, the conventional arginine-rich CPPs need micro-molar concentrations, thus, not suitable for delivery to the nano-molar modulators.

In previous studies, we synthesized the amphiphilic dimeric bundle peptide, LK-3, and successfully delivered nano-molar modulators into cells. LK-3 consists of two monomers (LK-2) linked by two disulfide bonds, and is the cell penetrating peptide that enters the cell at the nano-molar concentration in eukaryotic cells.[5] On the helical wheel projection, the 16-amino-acid-long (16-mer) amphipathic monomer has a hydrophobic face consisting of Leucine (Leu) and a positively charged hydrophilic face consisting of Lysine (Lys). Two monomers are bound by two disulfide bonds of two cysteines (Cys) located between the hydrophilic side and the hydrophobic side. Leu on the hydrophobic face can interact with Leu of other molecules or hydrophobic cargo, and the property of amphipathicity is an important factor in cell permeability.[6] However, LK-3 is cytotoxic as well as difficult to synthesize for industrial use.

One of the major problems in the use of CPP is the harmony between cell permeability and cell toxicity. Because CPP itself enters the cell, it can cause
cytotoxicity at high concentrations. To minimize the toxicity of CPPs, the concentration used for delivering modulator should be much lower than the toxic concentration. Therefore, it is important to develop low-toxicity CPPs and solve the toxicity limitations of CPP applications.

LK-2, a monomer of the model peptide LK-3, is 16-mer. If the lengths are different, the shape of the monomer may vary and may also affect cell permeability. Although it is considered that the longer the monomer length of the dimeric bundle peptide, the better the cell permeability, however, the changes in cell permeability in relation to peptide length have not been studied yet in dimeric peptide bound by two disulfide bonds. On the other hand, for industrial use, it is important to reduce the amount of raw materials and to increase the yield by producing in fewer steps. One more important point in the optimization process is to reduce the time required for dimerization (In case of LK-3, it takes more than three days.)

In this study, we modified the model peptide, LK-3. We designed and synthesized the second generation CPPs by adjusting lengths and changing residues in hydrophilic and hydrophobic faces of the amphipathic monomer peptide. The purpose of optimization was to generate peptides that can be generated economically, have low cell toxicity, and are capable of penetrating cell membrane to similar level. Dimerization kinetics was observed and cell toxicity was determined by WST-1 assay in HeLa cell. Cell penetration efficiency was measured by flow cytometry analysis of the degree of uptake into cells using fluorescence-labeled-peptides in HeLa cell. The drug delivery capacity was confirmed by conjugating methotrexate (MTX) to MDA-MB-231 cell, MTX-resistant cell. In addition, we examined the correlation between hydrophobicity and cell permeability and the effect of changes in hydrophilic face amino acids.
**Experimental Section**

1. **Peptide synthesis**

1.1 Synthesis of monomeric peptides

All peptides were synthesized by using a standard Fmoc solid phase synthesis methods with an SPS microwave peptide synthesizer (Discover, CEM). For peptide synthesis, 9-fluorenylmethyloxycarbonyl (Fmoc)-protected leucine, lysine, cysteine, alanine, glycine, isoleucine, phenylalanine, arginine and histidine were purchased from Novabiochem. Fmoc-protected homo cysteine was purchased from Bachem. Fmoc-protected neophentylglycine was purchased from Chem-Impex International.

Rink Amide MBHA resins (50 mg, 0.064 mmol/g, Novabiochem) were deprotected with 20 % piperidine in N,N-dimethylformamide (DMF, Avantor). For Coupling step, Fmoc-protected amino acid (6 eq.), benzotriazol-1-yloxytrpyrrolidinophosphonium hexafluorophosphate (PyBOP, 6 eq. Novabiochem) and N,N-diisopropylethylamine (DIPEA, 6 eq. Sigma) dissolved in DMF were placed in microwave vessel and reacted with peptide synthesizer. And then resin was washed using DMF and dichloromethane (DCM, Daejung). The deprotection and coupling steps were repeated sequentially. The confirmation of the reaction was monitored by TNBS (2,4,6-Trinitrobenzenesulfonic acid) test.

After the coupling of final amino acid at N-terminal, 20 % piperidine in DMF was treated for deprotection of Fmoc and resin was washed with DMF and DCM. For acetylation of N-terminal, mixture containing acetic anhydride (Sigma), 1-hydroxybenzotriazole hydrate (HOBt, Novabiochem) and 10% v/v DCM in DMF...
was used and reaction was performed on peptide synthesizer.

5(6)-Carboxytetramethylrhodamine (TAMRA, Sigma) fluorescence dye was conjugated to N-terminal of Fmoc deprotected peptide for flow cytometry analysis. For conjugation, 5(6)-TAMRA (2 eq.), O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, Novabiochem, 2eq.), HOBt (2 eq.) and DIPEA (4 eq.) were dissolved in DMF. The N-terminal deprotected resins were reacted with the mixture for 2 hours at room temperature.

Methotrexate (MTX) was conjugated to the N-terminus of Fmoc deprotected peptide for measuring MTX delivery capacity. For conjugation, the mixture of MTX (3 eq. Sigma), HOBt (1.5 eq.), HCTU (3 eq.), DIPEA (6 eq.) in DMF was incubated with shaking at room temperature for 10 min. And next, the reaction of N-terminal deprotected resins and the mixture was performed for 2 h at room temperature.

For cleavage of synthesized peptide, cleavage solution containing 940 µL of Trifluoroacetic acid (TFA, Sigma), 10 µL of Triisopropylsilane (TIS, Sigma), 25 µL of EDT (Ethanedithiol, Sigma) and 25 µL of distilled water was used and shaken for 2 hours. And then, resins were filtered and washed with TFA. Separated peptide was precipitated using mixture combination of n-hexane (Daejung) and diethyl ether (Avantor). After centrifugation, the supernatant was removed. These steps were repeated for three times and then the peptide sedimentation was dissolved in methyl sulfoxide (DMSO, Sigma). Synthesized peptide was purified by HPLC using Zorbax C_{18} column (3.5 μm, 4.6 x 150 mm) as a stationary phase. For mobile phase, mixture of buffer A (distilled water with 0.1 % v/v TFA) and buffer B (acetonitrile with 0.1 % v/v TFA) was used at a flow rate of 1 mL/min. (Condition of mobile phase: 0-5 min, 5 % of buffer B followed by linear gradient
5-70 % of buffer B over 25 min). Purified peptides were confirmed by using a Voyager™ MALDI-TOF mass spectrometer. The HPLC chromatograms and MALDI-MS data were shown in Figure 3. After purification, the peptides were lyophilized by freeze dryer (Operon). Concentration of peptide including acetyl capped, fluorescence labeled and Methotrexate conjugated peptide was calculated by a spectrometer (Direct Detect™ spectrometer, Merk millipore).

1.2 Peptides dimer formation (dimerization)

Dimeric peptides were formed by air oxidation process. Generally, purified cysteine containing monomeric peptide was dissolved in 0.1 M ammonium bicarbonate to give final concentration of 1 mM, and the mixture was incubated in the atmosphere until the reaction was complete. The reaction completion was monitored by HPLC and mass detection. After the reaction, dimeric peptides were purified by HPLC and lyophilized as described above. For fluorescence labeled dimeric peptides, 5-TAMRA was labeled on only one strand.

2. Kinetic study of dimerization

The dimerization reaction was performed at the final monomeric peptide concentration of 1mM and the dimerization method was as described above. The decrease in the amount of monomer was monitored by HPLC and calculated as area ratio. Observations were performed immediately after the reaction (0 hour) and at 2, 4, 8, and 24 hours after the reaction. If the reaction was not complete, concentration of monomer was observed for several more days.
3. Cell culture

HeLa cells (ATCC), the human cervical cancer cell line, were cultured in Dulbecco’s modified Eagle’s medium (DMEM, HyClone, Thermo Fisher Scientific) containing 10% (v/v) of fetal bovine serum (FBS, HyClone, Thermo Fisher Scientific) and 1% (v/v) of Penicillin/Streptomycin (Pen-Strep, HyClone, Thermo Fisher Scientific) at 37 °C in the presence of 5% CO₂.

MDA-MB-231 cells, the human breast cancer cell line, were cultured under the same conditions as HeLa cells.

4. Flow cytometry analysis

HeLa cells (5x10⁴ cells/well) were seeded in 24-well plate in complete media (DMEM containing 10% FBS and 1% Pen-Strep). After 24 h incubation, cultured media was removed and cells were treated with 5-TAMRA labeled peptides at various concentrations in fresh complete media. After 24 h incubation, cells were washed with Phosphate Buffered Saline (PBS, HyClone) and trypsinized. Detached cells were collected in microcentrifuge tubes and centrifuged at 14,000 rpm for 2 min, and resuspended in PBS.

Cellular uptake of peptides was analyzed by using a flow cytometer (BD Accuri C6, BD Biosciences). Cells were analyzed at 488 nm laser and 1 x 10⁴ cells were assessed for each sample and dead cells were excluded from the analysis. Cellular uptake of peptides was analyzed by FACS and calculated using TAMRA positive cells (%) and Mean fluorescence.
5. Cell viability assay (WST-1 assay)

HeLa cells (1 x 10^4 cells/well) were seeded in 96-well plate in complete DMEM media. After 24 h incubation, cultured media was removed and cells were treated with peptides at various concentrations in fresh complete media. After 24 h incubation, WST-1 reagent (EZ-CYTOX, Dogen) was added to the each well and incubated for 30 min at 37 °C in the incubator. The absorbance of each well was measured at 450 nm and 700 nm (reference) by using a 96-well microplate reader (UVM 340, Biochrom).

MDA-MB-231 cells were seeded in 96-well plate at a density of 2.5 x 10^3 cells per well in complete DMEM media. After 6 h incubation, cultured media was removed and cells were treated with peptides at various concentrations in fresh complete media. After 48 h incubation, WST-1 reagent was added to the each well and incubated for 40 min at 37 °C in the incubator. The absorbance of each well was measured at 450 nm and 700 nm (reference) by using a 96-well microplate reader.
Results and Discussion

1. Design, synthesis and feature of peptides

The sequence of the peptides was designed by modifying each of the three features of the first generation CPP, LK-3. For the purpose of reducing cytotoxicity, the length of the monomer peptide was shortened, and longer monomers were also synthesized for comparison. The helical wheel diagram of the dimer peptide composed of a 10-amino-acid-long monomer (10-mer) is shown in Figure 1. The dimer peptides oxidized by air oxidation condition are an antiparallel structure.[6] As seen from the side, the dimer peptides have a little crossed shape rather than a completely parallel straight line as guessed from the Figure 2. To synthesize the second generation CPPs maintaining dimeric amphipathic peptide characteristics, a peptide library was designed by substituting residues constituting the hydrophilic and hydrophobic faces with different amino acids. Lysine (Lys) was substituted with positively hydrophilic amino acids such as arginine (Arg) and histidine (His). And leucine (Leu) was substituted with hydrophobic amino acids such as isoleucine (Ile), phenylalanine (Phe), valine (Val) and neopentylglycine (Npg). The structure of each amino acid is shown in Figure 2. In the dimer structure, each cysteine (Cys) is bonded to form a disulfide bond between the two monomers, thus two monomers are separated by four atoms (CSSC). Are the cell permeability and complexing with modulator at the optimal level when the spacer length is four atoms? To research this, homocysteine (Hcy) can be used instead of Cys to adjust the length of spacer between two monomer peptide skeletons to five (CCSSC or CSSCC) or six (CCSSCC). Such adjustment may be helpful for maximizing the cognition of hydrophobic molecules, and thus the formation rate of dimer, the cell
permeability, and the complex formation with the modulator may vary. The sequences of monomer are shown in Table 1 and the LC chromatograms and molecular weight of the prepared dimer peptides are shown in Figure 3. To measure cell permeability, the peptide was labeled with fluorescence and analyzed by flow cytometry analysis.

**Figure 1.** The schematic structure of the dimer peptide composed of a 10-mer

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence of peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY10</td>
<td>Ac-XCYXXYYXYC-NH₂</td>
</tr>
<tr>
<td>diXY10</td>
<td>Ac-XCYXXYYXYC-NH₂</td>
</tr>
<tr>
<td></td>
<td>NH₂-YCXYYXYXXYCX-Ac</td>
</tr>
</tbody>
</table>

X = Hydrophobic amino acid (Leu, Ile, Val, Phe, Npg)

Y = Hydrophilic amino acid (Lys, Arg, His)

C = Cystein derivative (Cys, Hcy)
Figure 2. The structures of amino acids

Leu  Ile  Val  Phe

Npg  Lys  Arg  His

Cys  Hcy
Table 1. The sequences of monomer peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence (monomer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LK8</td>
<td>Ac-CKLLKLC-NH₂</td>
</tr>
<tr>
<td>LK10</td>
<td>Ac-LCKLLKLC-KNH₂</td>
</tr>
<tr>
<td>LK12</td>
<td>Ac-KLCKLLKLC-KNH₂</td>
</tr>
<tr>
<td>LK14</td>
<td>Ac-KKLCKLLKLC-KNH₂</td>
</tr>
<tr>
<td>LK16</td>
<td>Ac-LKKLCKLLKLC-KNH₂</td>
</tr>
<tr>
<td>LK18</td>
<td>Ac-LLKKLCKLLKLC-KNH₂</td>
</tr>
<tr>
<td>LK-2</td>
<td>Ac-LKKLCKLLKLC-KLAG-NH₂</td>
</tr>
<tr>
<td>IK10</td>
<td>Ac-ICKI1KIC-NH₂</td>
</tr>
<tr>
<td>VK10</td>
<td>Ac-VCKVKKVCK-NH₂</td>
</tr>
<tr>
<td>FK10</td>
<td>Ac-FCKFCK-FCK-NH₂</td>
</tr>
<tr>
<td>NpgK10</td>
<td>Ac-NpgCKNpgNpgKK-NH₂</td>
</tr>
<tr>
<td>LR10</td>
<td>Ac-LCRLLRLRCR-NH₂</td>
</tr>
<tr>
<td>IR10</td>
<td>Ac-ICRIIRICR-NH₂</td>
</tr>
<tr>
<td>FR10</td>
<td>Ac-FCRFFRFCR-NH₂</td>
</tr>
<tr>
<td>NpgR10</td>
<td>Ac-NpgCRNpgNpgRRNpgCR-NH₂</td>
</tr>
<tr>
<td>LH10</td>
<td>Ac-LCHLLHHCH-NH₂</td>
</tr>
<tr>
<td>IH10</td>
<td>Ac-I1CHIHIC-NH₂</td>
</tr>
<tr>
<td>VH10</td>
<td>Ac-VCHVHHVCH-NH₂</td>
</tr>
<tr>
<td>FH10</td>
<td>Ac-FCHFHHFCH-NH₂</td>
</tr>
<tr>
<td>NpgH10</td>
<td>Ac-NpgCHNpgNpgHHNpgCH-NH₂</td>
</tr>
<tr>
<td>LK10-C9Hcy</td>
<td>Ac-LCKLKKLHCyK-NH₂</td>
</tr>
<tr>
<td>LK10-C2Hcy/C9Hcy</td>
<td>Ac-LHCyKLLKKLHCyK-NH₂</td>
</tr>
<tr>
<td>LR10-C2Hcy</td>
<td>Ac-LCRLLRLHCyR-NH₂</td>
</tr>
<tr>
<td>LR10-C9Hcy</td>
<td>Ac-LCRLLRLHCyR-NH₂</td>
</tr>
<tr>
<td>LR10-C2Hcy/C9Hcy</td>
<td>Ac-LHCyRLRRLHCyR-NH₂</td>
</tr>
<tr>
<td>NpgR10-C2Hcy</td>
<td>Ac-NpgCRNpgNpgRRNpgHCyR-NH₂</td>
</tr>
<tr>
<td>NpgR10-C9Hcy</td>
<td>Ac-NpgCRNpgNpgRRNpgHCyR-NH₂</td>
</tr>
<tr>
<td>NpgR10-C2Hcy/C9Hcy</td>
<td>Ac-NpgHCyRNpgNpgRRNpgHCyR-NH₂</td>
</tr>
<tr>
<td>LR10-C2L</td>
<td>Ac-LLRLLRLRCR-NH₂</td>
</tr>
<tr>
<td>LR10-C9L</td>
<td>Ac-LRLLRLRLRC-NH₂</td>
</tr>
</tbody>
</table>
Figure 3. HPLC chromatograms and molecular weight of dimer peptides

**diLK10**, t<sub>R</sub>: 21.659 min. MS [M+H]<sup>+</sup>: 2456.51 (calcd), 2458.295 (obsd)

**diIK10**, t<sub>R</sub>: 22.307 min. MS [M+H]<sup>+</sup>: 2456.51 (calcd), 2457.508 (obsd)

**diFK10**, t<sub>R</sub>: 21.205 min. MS [M+H]<sup>+</sup>: 2728.39 (calcd), 2730.092 (obsd)

**diNpgK10**, t<sub>R</sub>: 24.154 min. MS [M+H]<sup>+</sup>: 2568.64 (calcd), 2569.673 (obsd)
diLR10, t<sub>R</sub>: 22.631 min. MS [M+H]<sup>+</sup>: 2680.56 (calcd), 2683.512 (obsd*)

diR10, t<sub>R</sub>: 23.268 min. MS [M+H]<sup>+</sup>: 2680.56 (calcd), 2679.098 (obsd*)

diFR10, t<sub>R</sub>: 21.941 min. MS [M+H]<sup>+</sup>: 2952.44 (calcd), 2954.309 (obsd*)

diNpgR10, t<sub>R</sub>: 21.659 min. MS [M+H]<sup>+</sup>: 2792.69 (calcd), 2793.335 (obsd*)
**LK-3**, $t_R$: 24.556 min. MS [M+H]$^+$: 3677.35 (calcd), 3679.890 (obsd)

**diLR10-C2Hcy**, $t_R$: 24.288 min. MS [M+H]$^+$: 2708.59 (calcd), 2712.412 (obsd*)

**diLR10-C9Hcy**, $t_R$: 25.093 min. MS [M+H]$^+$: 2708.59 (calcd), 2708.529 (obsd*)
diLR10-C2Hcy/C9Hcy, $t_R : 22.041$ min. MS [M+H]$^+ : 2736.62$ (calcd), 2734.52 (obsd)

- The purity of all peptides is over 95%.

- $t_R$: Retention time of peptides. Conditions of HPLC elute: buffer A (0.1 \% v/v TFA in water) and buffer B (0.1 \% v/v TFA in acetonitrile); 0-5 \% (B \%) over 5 min, 5-70 \% (B \%) over 25 min; detected at UV, 220nm; column, Zorbax C18 column (3.5 \mu m, 4.6 x 150 mm)

- MS of peptides was measured using a Voyager™ MALDI-TOF mass spectrometer (Applied Biosystems). Usually the RP method is used, and when MS is measured by LP method, it is indicated by obsd *.
2. Adjustment of monomer peptide length

In order to diversify the length of the monomer peptide while maintaining the characteristics of LK-2, the peptides were shortened or extended at both ends of the sequence (Table 1, LK8 - LK18). LK16 is a sequence in which the AG at the C terminal of LK-2 is substituted with LK. Except for LK12, the rate of monomer disappearance is the same as the rate of dimer formation, which was expressed as the change in monomeric peptide concentration (Figure 4). Surprisingly, the dimerization rate of LK10, the short 10-mer, was the fastest among other peptides and four times faster than LK-2. Meanwhile the dimers with shorter or longer length were slower. Unlike other peptides, LK12 was rapidly aggregated. Also, LK12 formed various oligopeptides (i.e. trimer, tetramer) rather than forming only dimer, for that reason, the yield of the dimer was substantially very low. Therefore, the rate of dimerization was the fastest in LK10 and more than 90% of the reaction was terminated within 8 hours, while the time required for LK16 or LK18 was more than 10 days. The shorter length, LK8, was rather slower than LK10.

Next, the cell permeability was observed by using a fluorescence-labeled dimer peptide. The first generation CPP, LK-3, had the best cell permeability while other peptides’ cell permeability decreased as their length decrease (Figure 5). Although LK-3 possessed cell permeability at a single unit of nanomolar concentration, the proportion of fluorescent positive cell is dramatically enhanced by the concentration of CPP. diLK10, short in length, has low cell permeability at a low nanomolar concentration, nevertheless it has a comparable cell permeability to that of LK-3 at several tens of nanomolar concentration. The fluorescent positive cell percent of R0 is only 50% at that concentration.
In fact, the concentration at which a modulator can be delivered into a cell can reach tens or hundreds of nano-molar concentration. At that concentration, the cell permeability of diLK10 is sufficient. The cytotoxicity to be mentioned later is also lower in diLK10 than in LK-3. In addition, as a delivery tool, economic considerations such as shortening the synthesis process, reducing the raw materials, and speeding up the formation of the dimer should be considered. Therefore, diLK10 was determined to be the optimized length.
Figure 4. Dimerization rate as changes in monomer length

\[ [A] = [A]_0 e^{-kt} \]

<table>
<thead>
<tr>
<th></th>
<th>LK8</th>
<th>LK10</th>
<th>LK12</th>
<th>LK14</th>
<th>LK-2</th>
<th>LK16</th>
<th>LK18</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k ) (hour(^{-1}))</td>
<td>0.012</td>
<td>0.27</td>
<td>n.d.*</td>
<td>0.10</td>
<td>4.0E-06</td>
<td>7.9E-06</td>
<td>6.4E-05</td>
</tr>
</tbody>
</table>

* n.d. represents not determined.
Figure 5. Cell permeability as changes in monomer length
3. Modification of residues in hydrophilic and hydrophobic face

3.1. Kinetic study

To maintain the amphiphilic feature of LK-3, Leu was replaced with other hydrophobic amino acids (Ile, Val, Phe, Npg) and Lys was replaced with other positively hydrophilic amino acids (Arg, His). We synthesized each of the 15 peptides (Table 1, LK10, IK10 – NpgH10). Comparing the dimerization rates of the 15 monomeric peptides synthesized, there was significant difference according to the change of the hydrophilic surface (Figure 6). The rate constant (k) was the largest at Arg \( (k = 0.154 \text{ – } 1.10) \), followed by Lys \( (k = 0.0186 \text{ – } 0.377) \), and the dimerization reaction of His \( (K = 0.0000165 \text{ – } 0.0182) \) was very slow. There was no significant difference in the hydrophobic aspect, except for Npg. It is interesting that the most hydrophobic Npg shows the slowest formation rate of the dimer. In the case of the Val-containing monomer, separation of the dimer was not easy due to problems such as formation of various oligomers or formation of kink (intramolecular disulfide bond in monomer) regardless of any amino acid on the hydrophilic surface. In the case of VR10 (data not shown), the monomer and kink appeared at more than three sites on liquid chromatogram and their morphology is probably not stable. IH10 (data not shown) was aggregated and samples could not be taken uniformly, thus measuring the dimerization rate was impossible.

The process of dimer formation can be considered as two steps: an intermolecular reaction (a first disulfide bond between two monomers) and an intramolecular reaction (a second disulfide bond). To observe the dimer formation process in detail, it was necessary to confirm the rate of dimerization in which only
one disulfide bond is formed. LR10-C2L and LR10-C9L were synthesized by replacing the two Cys of LR10 with Leu, respectively (Table 1), and the dimer formation rate was analyzed at a slightly lower concentration (0.75 mM of the monomer peptide). Interestingly, the dimerization rate of a monomer with one Cys was much faster than that of a monomer with two Cys (Figure 7). This means that the rate determining step of the dimeric bundle peptide formation is the step of forming the second disulfide bond, which is an intramolecular reaction. Thus, the rate of dimerization is determined by whether the temporary position is capable of forming the second disulfide bond well.

3.2. Cell penetration efficiency

Comparing the cell permeability of the dimer peptides, there were significant differences according to the change of the hydrophilic amino acid (Figure 8). The mean fluorescence intensity (at 62.5 nM) of the Arg-containing peptides were 10-fold stronger than that of His, and the Lys-containing peptides were 3-fold stronger than His. That is, the cell permeability was the best with Arg, followed by Lys, and was lowest when His was contained.

In the change of the hydrophobic face, Npg is particularly superior. diNpgR10 had a cell permeability comparable to that of LK-3 consisting of 16 amino acids, and the mean fluorescence intensity was 20 times higher than that of diLH10 at 62.5 nM, followed by Leu / Phe / Ile containing peptides. On the other hand, Npg was relatively well aggregated in the process of dimerization. This may be evidence that the cell permeability is related to the increase in the hydrophobicity of the peptide obtained as a result of increased hydrophobicity of the side chain.
Figure 6. Dimerization rate with changes of the hydrophilic and hydrophobic face.
Figure 7. Dimerization rate of a monomer with peptide containing one Cys

![Graph showing dimerization rate](image)

Figure 8. Cell permeability according to the type of amino acid

![Graph showing cell permeability](image)
Figure 9. Ratio of mean fluorescence intensity (at 62.5 nM)
4. Adjustment of spacer length

The third structural feature of the model peptide LK-3 is that it consists of two disulfide bonds. The length between two monomers could be controlled using homocysteine (Hcy), which is a homologue of the cysteine (Cys), differing by an additional methylene bridge (-CH₂-). Since there are two Cys in the monomer XY10, a monomer containing 0, 1 or 2 Hcy is possible. There are four atoms (CSSC) between two monomers in diXY10, and six atoms (CCSSCC) in diXY10-C2Hcy/C9Hcy. Monomers with one Hcy can be XY10-C2Hcy or XY10-C9Hcy depending on the position in the sequence. It was previously confirmed that an antiparallel dimer is formed under air oxidation condition.[6] Therefore, when XY10-C9Hcy (or XY10-C2Hcy) is oxidized under the same conditions, the Cys of the one monomer and the Hcy of the other monomer form disulfide bonds and length of spacer is five atoms (CSSCC or CCSSC). For the sake of understanding, the scheme of dimerization is shown in Figure 10.

We synthesized each of the eight peptides (Table1, LK10-C9Hcy - NpgR10-C2Hcy/C9Hcy). As we measured the cell permeability by modifying the dimer spacing, it was greatest when the length of spacer was 5 or 4 (Figure 11). The same tendency was observed when the amino acids constituting the monomer were different. In addition, the bigger the cell permeability of diXY10 (four atoms), the smaller the gap between the five atoms (diXY10-C2Hcy or diXY10-C9Hcy) and the four atoms. In comparison of cytotoxicity, four or five atoms cases possess low toxicities (Figure 12). Thus, the optimal length of spacer between the two monomers forming the dimer was five, and this result was consistent with the results of LK-3.[6]
Figure 10. The scheme of dimerization containing Hcy
Figure 11. Cell permeability according to length of spacer

![Cell permeability graph](image)

Figure 12. Cell toxicity according to length of spacer

![Cell toxicity graph](image)
5. Tendency between cell permeability and amino acids

In order to observe the tendency of the cell permeability of the dimeric peptide, the hydrophobicity of the dimer was determined by the retention time of HPLC. The retention time is linearly related to the increase of the hydrophobicity since the gradient of the solvent is linearly increased in the measured range. Cell permeability is directly correlated with each dimer and the correlation coefficient of $R^2$ is 0.8193 (Figure 13).

According to the comprehensive review of the changes in hydrophilicity, Arg has advantages over Lys in terms of dimerization rate, cell permeability, and cytotoxicity (Figure 14). More research is needed on the cause, but it may be related to the bidentate hydrogen bonds of the guanidine head group of Arg. Interaction with the negatively charged carboxylic, sulfate, and phosphate groups of cell membrane constituents leads to cellular internalization of CPPs. [7] On the other hand, His exhibited remarkably slow dimerization rate and cell permeability and showed a large difference compared to Arg or Lys. diLH10 shows little cytotoxicity at 40 μM, which is associated with little cell permeability. The pK$_a$ of the side chain is Arg (12.5) > Lys (10.5) > His (6.0), and the low cell permeability of His can be considered as a lack of positive charged property. This tendency, depending on the modification of amino acids, was not observed in LK-3,[6] and the research significance of the 10-mer series can be found.
**Figure 13.** Hydrophobicity and cell permeability

**Figure 14.** Comprehensive review of the changes in hydrophilicity

* diLH10 is non-toxic even at 40 μM (Data not shown).
6. Biological application using CPPs

To observe drug delivery capacity of the peptides, we used methotrexate (MTX) as a drug. MTX is an antitumor agent with a molecular weight of 454.44 g/mol that has been used widely in cancer therapy. Since MTX is an analogue of folic acid and its mechanism is involved in metabolism, its use in high concentration and low selectivity has serious risk of side effects. Thus, conjugating drug to the CPPs can be one of strategy to overcome these problems. In this experiment, we used MDA-MB-231 cell line, which is the breast cancer cells of Homo sapiens, because they are resistant to MTX (Table 2). Comparing with NIH-3T3 cell line (normal cells) and HeLa cell line (cancer cells), the IC50 of MTX in MDA-MB-231 cell line was 30-fold higher.

We conjugated MTX to the N-terminus of XY10 to synthesize MTX XY10 and dimerized MTX XY10 to make MTX diXY10. The IC50 of the MXT-conjugated peptide in MDA-MB-231 cells was 0.0139 - 0.0459, which was significantly lower than that of MTX, and the toxicity of the peptide itself was very low at IC50 3.41 - 13.7 (Figure 15). That is, CPPs delivered MTX into cells and increased MTX efficacy, and it is not because of the peptide’s self-toxicity. LK-3 has the best drug delivery capacity, but diNpgK10 also reduces the IC50 of MTX by 40-fold, which is comparable with LK-3 at 1.77 times. Other 10-mers have advantages over LK-3 because of their faster dimerization rate and lower toxicity to be mentioned later.
Table 2. IC<sub>50</sub> of MTX in NIH-3T3, HeLa and MDA-MB-231 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NIH-3T3</th>
<th>HeLa</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; of MTX (μM)</td>
<td>0.037</td>
<td>~0.031</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Figure 15. Cell viability of MDA-MB-231 cells

<table>
<thead>
<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>MTX</th>
<th>LK-3</th>
<th>diLK10</th>
<th>diLR10</th>
<th>diNpgK10</th>
<th>diNpgR10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; of MTX conjugated peptide (ratio)</td>
<td>0.972</td>
<td>~0.0139</td>
<td>0.0459</td>
<td>0.0401</td>
<td>0.0246</td>
<td>0.0316</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>(69.7)</td>
<td>(1.00)</td>
<td>(3.30)</td>
<td>(2.88)</td>
<td>(1.77)</td>
<td>(2.27)</td>
</tr>
<tr>
<td>Therapeutic index*</td>
<td>-</td>
<td>244</td>
<td>298</td>
<td>222</td>
<td>237</td>
<td>355</td>
</tr>
</tbody>
</table>

* Therapeutic index = IC<sub>50</sub> of Ac capping peptide / IC<sub>50</sub> of MTX conjugated peptide
7. Cytotoxicity

We observed the cytotoxicity of the peptide itself and all diXY10s showed lower toxicity than LK-3 in HeLa cell (Figure 16). Thus, we have achieved the initial purpose of alleviating cytotoxicity. Especially, diLR10 showed a very low cytotoxicity and the IC$_{50}$ of diLR10 is 4.3 times that of LK-3. Overall, the IC$_{50}$ of the Arg series is greater than that of Lys and all dimers show sufficiently low toxicity for use. This is because the micro-molar concentration at which the toxicity appears is much higher than the nano-molar concentration at which the dimers permeate the cell and deliver the drug.

Figure 16. Cell viability of the second generation CPPs

<table>
<thead>
<tr>
<th></th>
<th>LK-3</th>
<th>diLK10</th>
<th>diIK10</th>
<th>diFK10</th>
<th>diNpgK10</th>
<th>diLR10</th>
<th>diR10</th>
<th>diFR10</th>
<th>diNpgR10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ (μM)</td>
<td>4.44</td>
<td>12.4</td>
<td>11.4</td>
<td>9.36</td>
<td>10.3</td>
<td>18.9</td>
<td>14.4</td>
<td>18.2</td>
<td>13.8</td>
</tr>
<tr>
<td>ratio</td>
<td>1.00</td>
<td>2.79</td>
<td>2.57</td>
<td>2.11</td>
<td>2.32</td>
<td>4.26</td>
<td>3.24</td>
<td>4.10</td>
<td>3.11</td>
</tr>
</tbody>
</table>
Conclusion

We aimed to find an optimized CPP that show the lower toxicity and can be manufactured more economically than the first-generation dimeric bundle amphipathic CPP, LK-3. We designed and synthesized second-generation CPPs by adjusting three factors; length, amino acid variation and length of spacer. First, by adjusting the length, we found out that the 10-mer had sufficient cell permeability, low toxicity, and fast dimerization rate. Thus, the length of the monomer is fixed at 10 amino acids. Second, by changing the kind of amino acid, we found out the optimized CPP; diNpgR10, diNpgK10 and diLR10 are the best considering all factors. In addition, the cell permeability of the peptide was directly correlated with the hydrophobicity. And Arg-containing peptides were advantageous over Lys-containing peptides in terms of dimerization rate, cell permeability, and cytotoxicity. In order to observe the drug delivery capacity of CPP, MTX-resistant MDA-MB-131 cells were treated with MTX-conjugated peptides, and our CPPs increased the efficacy of MTX. Especially, the delivery capacity of diNpgK10 was the best among the 10-mer and comparable to LK-3. In addition, all diXY10 showed lower toxicity than LK-3. Taken together, we got a new delivery tool for nano-molar modulators in less-toxic and more-economic manner.

Since the dimeric bundle peptides are bound together by two disulfide bonds, they are expected to have a particular structure. Mutations in peptide length and amino acid will cause changes in structure and thus toxicity. Therefore, it is meaningful to synthesize various peptides and study their characteristics. In this study, especially, the adoption of arginine instead of lysine into the hydrophilic face
is interesting, because many types of CPP have multiple Arg. These Arg-rich peptides are mostly amorphous in structure, but interact with negatively charged substances (e.g. phosphate) on the cell surface. Therefore, it may be more advantageous than Lys in recognizing cell surface substances and in penetrating cell membrane.

The limitation of this study is that only the ability to deliver the peptide-conjugated drug is represented. When delivering drugs into cells, there are two methods; conjugated and non-conjugated with CPPs. Non-conjugated method means using complexes formed by simply mixing a peptide with a drug. Considering the production cost of CPPs, if CPPs can deliver the therapeutic agents by simply complexing (without conjugation), it will be advantageous not only economically but also convenience-wise; less therapeutic agent can be used and the synthesis process for conjugation between CPP and therapeutic agent can be skipped. Whether CPP with the best cell permeability is most capable of forming complexes with drugs is another issue, so further research is needed.
Reference


국문초록

세포 투과 펩타이드는 세포막을 투과하지 못하는 조절 물질을 전합
세포 안으로 전달하는 도구로서 많은 관심을 받아왔다. 그러나 기존의
아르기닌이 풍부한 세포 투과 펩타이드는 마이크로 물 농도를 필요로
하므로, 나노 물 수준에서 조절 물질을 전달하기에 적합하지 않다. 이것을
해결하기 위하여, 우리는 양친매성 단량체 형태의 이합체를 디자인하고
합성하였으며, 나노 물 수준에서 조절 물질을 성공적으로 전달했다.
그러나 아미노산의 길이가 16개인 양친매성 단량체로 만들어진 1세대
단량체 형태의 이합체 세포 투과 펩타이드인 LK-3는 세포 독성이 높고
산업적인 목적으로 합성하기에 어려움이 있다. 그래서 우리는 ‘펩타이드의
길이, 구성 아미노산의 종류, 단량체 사이의 간격’의 세 가지 요소를
조절하여 2세대 펩타이드를 설계하고 합성했습니다. 단량체의 길이를
조정한 결과, 단량체 펩타이드의 길이가 단지 아미노산 10개인 최적의
세포 투과 펩타이드 diLK10이 발견되었다. diLK10의 세포 투과 능력은
LK-3의 세포 투과 능력보다 낮지지만 폴리아르기닌 세포 투과 펩타이드인
R9보다 훨씬 좋으며, 실제적으로 약물 전달에 사용할 농도에서는 세포
투과 능력이 충분하다. 양친매성 단량체 펩타이드의 천수성 및 소수성
표면의 잔기를 각각 변화시켰을 때, 세포 투과성은 아르기닌 및 네오펜틸
글라이신 (Npg) 잔기가 천수성 표면 및 소수성 표면에 각각 존재할 때
가장 크게 나타났다. 이합체화 속도와 세포 투과 능력 모두를 고려할 때,
단량체 사이의 간격은 시스테린 대신 호모시스테인을 사용하여 미세하게 조정되었다. 단량체 사이 간격이 5개 원자일 때 세포 투과성이 좋았고, 독성이 6개 원자일 때 보다 낮았다. 약물 전달 능력을 확인하기 위해 메토트렉세이트 (MTX) 를 펩타이드에 결합시켰다. MTX 내성인 MDA-MB-231 세포에서 우리의 짧은 펩타이드는 MTX의 약효를 20배 이상 증가시켰다. 특히, diNpgK10은 MTX가 40배나 낮은 농도에서도 세포 내로 들어가도록 하였는데, 이를 LK-3와 유사한 수준이다. 또한 모든 diXY10은 LK-3보다 낮은 독성을 나타냈으며, 특히 diLR10의 IC₅₀은 거의 20 μM로, LK-3의 4.3배에 달한다. 따라서, 우리는 독성이 작고 경제적인 면을 고려하여 세포 안으로 들어갈 수 있는, 나노 물 조절 물질을 위한 새로운 전달 도구를 개발하였다.

주요어: 세포 투과 펩타이드, 양친매성, 이황화 결합, 다양화, 약물 전달, 메토트렉세이트

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