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An Amphipathic Structure of a Dipropylglycine-Containing Helical Peptide with Sufficient Length Enables Safe and Effective Intracellular siRNA Delivery

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Amphipathic peptides composed of cationic amino acids and hydrophobic amino acids have cell-penetrating ability and are often used as a delivery tool for membrane-impermeable compounds. Small interfering RNA (siRNAs) are one of the delivery targets for such cell-penetrating peptides (CPPs). Cationic CPPs can associate with anionic siRNAs by electrostatic interactions resulting in the formation of nano-sized complexes, which can deliver siRNAs intracellularly. CPPs containing unnatural amino acids offer promising tools to siRNA delivery. However, the detailed structure–activity relationship in siRNA delivery has been rarely studied. In the current study, we designed peptides containing dipropylglycine (Dpg) and explored the cellular uptake and cytotoxicity of peptide/siRNA complexes. The amphipathic structure of the peptides played a key role in complexation with siRNAs and intracellular siRNA delivery. In the amphipathic peptides, cellular uptake of siRNA increased with increasing peptide length, but cytotoxicity was reduced. A peptide containing four Dpg exhibited an effective gene-silencing effect with small amounts of peptides without cytotoxicity in medium containing serum. These findings will be helpful for the design of novel CPPs for siRNA delivery.

Key words cell-penetrating peptide, small interfering RNA (siRNA) delivery, helical structure, amphipathic structure, unnatural amino acid

Introduction

Small interfering RNAs (siRNAs) are an effective modality for RNA interference (RNAi) therapy. Recently, several siRNA therapeutics have been approved for clinical use.[1,2] Furthermore, siRNAs have been used as research tools to understand the function of genes and proteins in cells. However, siRNAs are easily degraded by nucleases in the body, in cell culture medium, and in cells. In addition, anionic and macromolecular siRNAs cannot permeate cell membranes. Thus, the development of drug delivery systems (DDSs) to protect and deliver siRNAs into the intracellular compartment is key for the success of siRNA therapeutics and siRNA research tools.

Cell-penetrating peptides (CPPs) are promising intracellular delivery tools for membrane-impermeable compounds and have been developed in clinical trials.[3–5] For example, Tat peptide was the first developed CPP, and its D-stereoisomer was used for the delivery of a functional peptide, which received orphan drug designation from the Food and Drug Administration and European Medicines Agency.[6] CPP-phosphorodiamidate morpholino oligomer conjugate, in which exon skipping phosphorodiamidate morpholino oligomer is used, has entered phase I/II clinical trials for Duchenne muscular dystrophy.[7] In this manner, CPPs are expected to have potentials as DDSs, whereas novel CPPs with high delivery efficiency and negligible toxicity are still being developed.

We have recently reported CPPs for the intracellular delivery of siRNAs.[8] In that report, amphipathic peptides were designed based on a model amphipathic peptide (MAP), α-helical 18-mer KLALKLALKALKAALKLA.[9–11] Including unnatural amino acids instead of alanine (Ala) allowed us to shorten the CPP length to a 12 mer with preserved α-helical structure. Among six peptides evaluated for the intracellular delivery of siRNAs, the peptide contained dipropylglycine (Dpg; Fig. 1A), which showed good gene-silencing effects with slight cytotoxicity. The exact sequence of its Dpg peptide is carboxyfluorescein (CF)-glycine (Gly)-(lysine (Lys)-Leu-Dpg-Leu)$_2$-Lys-Dpg-Leu-Lys-NH_2. CF was introduced into peptides as a hydrophobic fluorophore to monitor the intracellular behavior of peptides. Introduction of Dpg enhanced helicity and cell-penetrating ability of the peptide, resulting in effective transfection. The gene-silencing effect with the Dpg peptide was performed using a low siRNA concentration (10 nM) with slight cytotoxicity in experimental conditions using serum-free medium. In this study, peptide sequences for intracellular siRNA delivery were further optimized based on the Dpg peptide. Note that peptide-based intracellular siRNA delivery is often performed in serum-free medium.[12–15] Peptides composed of only natural α-amino acids are easily degraded by proteases and peptidases in serum, and thus, do not work. Furthermore, a large excess of peptides to siRNAs are more likely to be used for effective intracellular siRNA delivery.[15,16] We developed novel amphipathic CPPs delivering siRNA into the cells with small amounts of peptides and negligible cytotoxicity even in medium with serum by improving previous Dpg peptide.

In this study, we designed seven peptides without fluorophore (Fig. 1B). **Pep1** was a 13-mer peptide consisting of only natural α-amino acids, composed of an N-terminal 12-mer of

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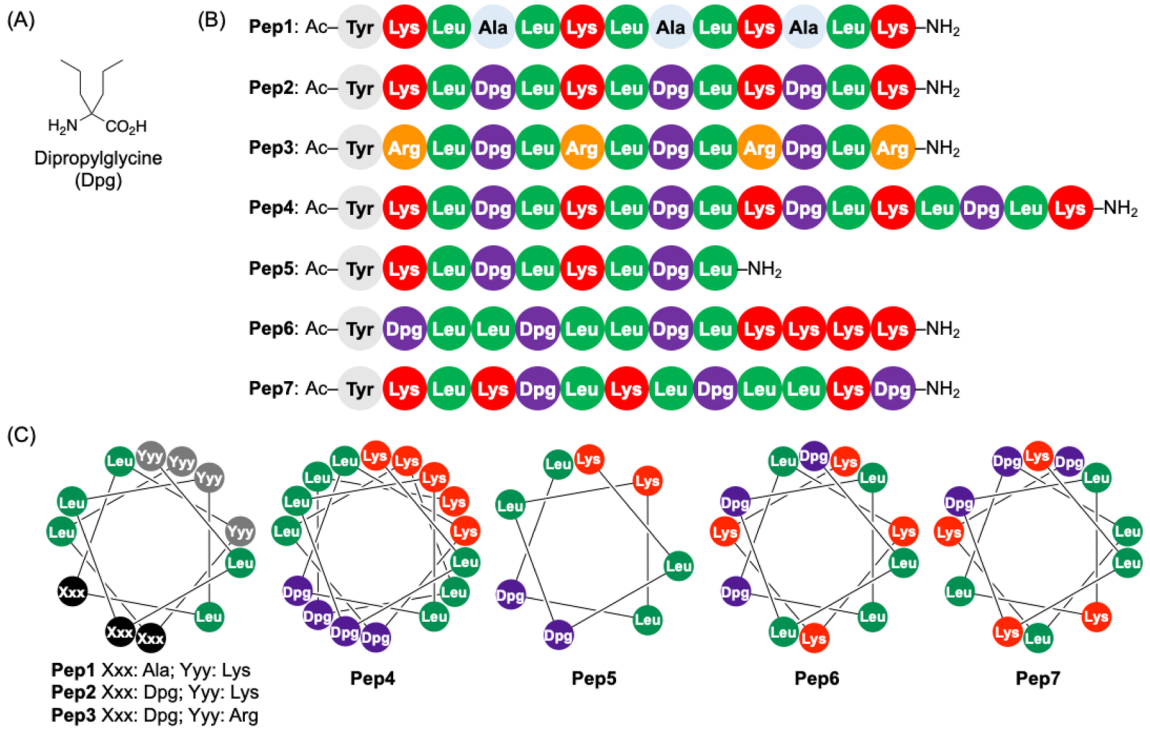


Fig. 1. (A) Structure of Dipropylglycine (Dpg); (B) Sequences of Peptides Described in the Current Study; (C) Schematic Illustration of the α-Helical Structures as Viewed along the Helical Axis

MAP and tyrosine (Tyr). **Pep2** had the same peptide sequence as a previous Dpg peptide[8] with N-terminal Ac-Tyr instead of CF-Gly. The replacement of Ala in **Pep1** with Dpg gave **Pep2**. The introduction of unnatural amino acid Dpg into the peptides was expected to acquire enzyme resistance.[17,18] In **Pep3**, Lys in **Pep2** were replaced with arginine (Arg) to evaluate the effect of cationic amino acids. **Pep4** had four additional amino acids compared with **Pep2**, whereas four amino acids were removed from **Pep2** to afford **Pep5**. Comparison of **Pep2**, **Pep4**, and **Pep5** revealed the effect of peptide length. **Pep1**–**Pep5** adopted an amphipathic structure when forming an α-helical structure (Fig. 1C). **Pep6** and **Pep7** had same number of each amino acid as **Pep2** but different sequences. Four cationic Lys were located in the C-terminus in **Pep6**, which formed a different type of an amphipathic structure compared with **Pep2. Pep7**, in which Lys existed randomly, but was unable to form an amphipathic structure even when adopting an α-helical structure. We evaluated the preferred secondary structures of peptides and the physicochemical properties of peptide/siRNA complexes followed by evaluation of intracellular siRNA delivery and cytotoxicity of the complexes. The current study about structure–activity relationship in siRNA delivery revealed that a Dpg-containing peptide **Pep4** formed an amphipathic α-helical structure and achieved effective intracellular siRNA delivery using small amounts of peptides with negligible cytotoxicity.

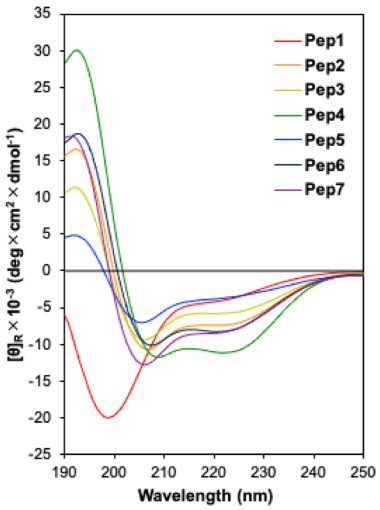
Results and Discussion

Characterization of Peptides Seven peptides, **Pep1**–**Pep7**, were synthesized using the microwave-assisted 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase method. Tyr was introduced at the N-terminus of peptides to determine the peptide concentration. The synthesized peptides were purified by reverse-phase HPLC (RP-HPLC) and characterized by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS). The purities of **Pep1**–**Pep7** were also confirmed by RP-HPLC as more than 96% (Supplementary Fig. S1).

Next, the preferred peptide secondary structures of **Pep1**–**Pep7** were evaluated by circular dichroism (CD) spectral measurements at a peptide concentration of 100 μM in the ultrapure water (Fig. 2). The CD spectrum of a peptide adopting a right-handed helical structure is known to show a positive maximum at around 192 nm and negative maxima at around 208 and 222 nm.[19–21] The helicity of each peptide was calculated from the value of $[\theta]_{222}$.[22] The type of helical structure, an α-helical structure or a 3_{10}-helical structure, can be distinguished from the value of $[\theta]_{222}/[\theta]_{208}$ (α-helical structure: $[\theta]_{222}/[\theta]_{208} \approx 1$; 3_{10}-helical structure: $[\theta]_{222}/[\theta]_{208} < 0.4$). Except for **Pep1**, **Pep2**–**Pep7** showed the spectra of a characteristic right-handed helical structure. The replacement of Ala with Dpg led to a dramatic change in peptide secondary structure to a helical structure (**Pep1** *vs.* **Pep2**) as reported previously.[8] A shorter peptide (**Pep5**) still maintained a helical structure by virtue of Dpg, though not an α-helical structure but a 3_{10}-helical structure. Elongation of the peptide sequence enhanced the helicity and preferred an α-helical structure over a 3_{10}-helical structure (**Pep2** *vs.* **Pep4** *vs.* **Pep5**). Almost no difference was brought by replacement of Lys with Arg (**Pep2** *vs.* **Pep3**). Peptides with the same amino acids but with different sequences showed similar CD spectra, indicating that the peptide sequences had a negligible effect on the peptide secondary structures (**Pep2** *vs.* **Pep6** *vs.* **Pep7**).

Physicochemical Properties of Peptide/siRNA Complexes Confirmation of peptide/siRNA complex formation was performed using gel retardation analysis at various N/P ratios,

(A)



(B)

Peptide	Helicity	$[\theta]_{222}/[\theta]_{208}$
Pep1	16%	n.d.
Pep2	29%	0.70
Pep3	22%	0.62
Pep4	38%	0.95
Pep5	19%	0.53
Pep6	32%	0.82
Pep7	32%	0.65

Fig. 2. (A) CD Spectra of **Pep1**–**Pep7** in Ultrapure Water; (B) CD Spectral Data of **Pep1**–**Pep7**

Peptide concentration: 100 μM. Helicity (%) was calculated as $100 \times [\theta]_{222}/[-40000 \times (1-2.5/n)]$, where n indicated the number of amino acids in the peptide sequence.[22]

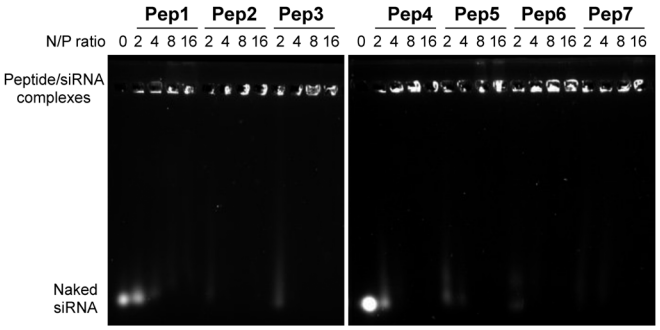


Fig. 3. Gel Retardation Analysis of **Pep1**–**Pep7**/siRNA Complexes with Various N/P Ratios

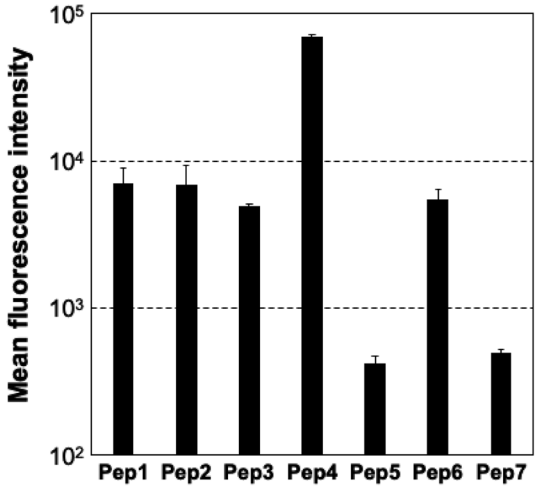


Fig. 4. Huh-7-Luc Cellular Uptake of Alexa488-siRNA Delivered by **Pep1**–**Pep7** (N/P Ratio : 4; siRNA Concentration: 100 nM; Incubation Time: 24 h)

Error bars represent the standard deviation, $n = 3$.

which were defined as the molar ratio of the amino or guanidino groups in peptides to the phosphate groups in siRNA (Fig. 3). All peptides showed free siRNA bands at N/P = 2, and **Pep1**, **Pep5**, and **Pep7** also showed them at N/P = 4. These results indicated that the complexation was completed at over N/P = 2 for **Pep2**–**Pep4** and **Pep6**, and at over N/P = 4 for **Pep1**, **Pep5**, and **Pep7. Pep1** did not form a helical structure and had low hydrophobicity compared with the other peptides because of having Ala instead of hydrophobic Dpg. **Pep5** had the shortest sequence of all the peptides. **Pep7** adopted a helical structure but did not form an amphipathic structure. These factors may have led to the low complexability of **Pep1**, **Pep5**, and **Pep7** with siRNA.

In order to obtain further insights into the physicochemical properties of peptide/siRNA complexes, the size of complexes was evaluated by dynamic light scattering (DLS) measurements (Supplementary Table S1). **Pep1** showed huge sizes at all N/P ratios compared with the other peptides, whereas **Pep2**–**Pep7** showed similar several hundred nanometer sizes at N/P = 4 and 8. These values were suitable for intracellular nano DDS use.[23] However, the polydispersity index (PDI) of each nano-sized complex was moderate or high far from monodispersity, implying the existence of variable sized complexes (from small to large) and/or rod-shaped complexes.

Intracellular Internalization of Peptide/siRNA Complexes To understand the cellular uptake amount of siRNA delivered by each peptide, flow cytometric analysis was performed using Alexa488-labeled siRNA (Alexa488-siRNA). From the viewpoints of complexation (Fig. 3) and moderate peptide concentration, N/P = 4 was adopted. Peptide/Alexa488-siRNA complexes prepared at N/P = 4 were incubated with Huh-7-Luc cells in medium containing 10% fetal bovine serum (FBS) at an siRNA concentration of 100 nM for 24 h. Figure 4 shows that the cellular uptake amounts of Alexa488-siRNA were much higher for **Pep4** ($p < 0.001$) and lower for **Pep5** and **Pep7** ($p < 0.05$) than **Pep1**–**Pep3** and **Pep6. Pep5** was shorter than the other peptides and showed weak associating ability with siRNA (Fig. 3), which may have led to low cellular uptake of siRNA. **Pep7** adopted a right-handed helical structure (Fig. 2) but did not form an amphipathic structure. These factors might have contributed to the low associating ability of **Pep7** not only to siRNA but also to cell membranes, resulting in low intracellular internalization of siRNA. The replacement of Dpg in **Pep2** with Ala (**Pep1**) and that of Lys

in **Pep2** with Arg (**Pep3**) had no effect on their siRNA intracellular delivery (**Pep1** *vs.* **Pep2** *vs.* **Pep3**). **Pep6** had four Lys residues at the C-terminus of its sequence, and it formed a different type of an amphipathic structure compared with **Pep2**. Thus, **Pep6** showed similar cellular uptake amounts of siRNA compared with **Pep2**.

In addition to flow cytometric analysis, fluorescence microscopic observation was performed to evaluate the intracellular behavior of siRNAs delivered by peptides (Fig. 5). Fluorophore Alexa488 (green) labeled siRNAs were monitored in Huh-7-Luc cells with staining of nuclei by Hoechst 33342 (blue). The results obtained here were roughly consistent with the results in Fig. 4. Bright green signals were observed in Huh-7-Luc cells treated with **Pep4**, and moderate ones were observed in those treated with **Pep1** and **Pep2**. On the other hand, **Pep5** and **Pep7** showed almost no signals.

RNAi Efficiency of Peptide/siRNA Complexes First, the RNAi efficiency was evaluated using peptide/siRNA complexes at various N/P ratios (Fig. 6A). An siRNA against the luciferase gene (Luc siRNA) was selected for this luminescence-based gene silencing assay and scrambled siRNA (Scr siRNA) was used as a control sequence for Luc siRNA. Peptide/siRNA complex solution was added to the luciferase-expressing human hepatoma Huh-7-Luc cells with an siRNA concentration of 100 nM. The luciferase assay was carried out after incubation for 24 h for complexes with cells in medium containing 10% FBS. At N/P = 2, all peptides exhibited similar luciferase activities of Huh-7-Luc cells in both Luc siRNA and Scr siRNA treatments, and hence no gene-silencing effect was observed. The luciferase activities of Huh-7-Luc cells treated with **Pep4**/Luc siRNA complexes decreased by 70% at N/P = 4 and 85% at N/P = 8 compared with those with Pep4/Scr siRNA complexes. **Pep6** showed a slight gene-silencing effect at N/P = 8 (30% knockdown). On the other hand, **Pep5** treatments dramatically reduced the luciferase activities both in Luc siRNA and Scr siRNA with an increase in N/P ratios, implying that **Pep5** was highly toxic. In addition, **Pep2** and **Pep7**/Scr siRNA complexes at N/P = 8 showed slight decreases in luciferase activities compared with non-treated Huh-7-Luc cells (cells only); therefore, **Pep2** and **Pep7** seemed to be slightly toxic. The experimental conditions (incubation time and siRNA concentration) used here were same as those in Figs. 4 and 5. The good gene-silencing effect of **Pep4** seemed to be attributed to high cellular uptake of siRNA. Commercially available transfection reagent Lipofectamine 3000 did not work in these experimental conditions.

Second, time-dependent RNAi efficiency was evaluated using peptide/siRNA complexes prepared at N/P = 4 and with an siRNA concentration of 100 nM (Fig. 6B). The peptide concentrations with the conditions of N/P = 4 and 100 nM siRNA were 4.2 μM for **Pep1**–**Pep3**, **Pep6**, and **Pep7**, 3.4 μM for **Pep4**, and 8.4 μM for **Pep5**, respectively. **Pep4** showed definite knockdown effects at all incubation times, whereas other peptides did not show significant knockdown effects. The luciferase activities of **Pep5** treatment were very low compared with cells only assays at all incubation times. **Pep4** may work at the acceptable concentration (3.4 μM).

Third, the effect of siRNA concentration on RNAi efficiency was evaluated (Supplementary Fig. S2). Peptide/siRNA complexes were prepared at N/P = 4 and incubated with Huh-7-Luc cells for 24 h. In **Pep4**, a slight gene-silencing effect (approximately 40% knockdown) was observed even at 50 nM siRNA (peptide concentration: 1.7 μM), and a considerable gene-silencing effect (approximately 80% knockdown) was observed at 200 nM siRNA (peptide concentration: 6.8 μM). **Pep3** and **Pep6** showed significant gene-silencing effects at an siRNA concentration of 200 nM; however, the luciferase activity of **Pep3**/Scr siRNA complex treatment also decreased. Not only **Pep3** but also **Pep2**, **Pep5**, and **Pep7**/Scr siRNA complex treatments at 200 nM siRNA showed drastically decreased luciferase activity, suggesting that they exhibited cytotoxicities.

A previous report with a **Pep2** analogue, in which CF-Gly was replaced with N-terminal Tyr, worked at a peptide/siRNA concentration of 1.0 μM/10 nM, at a low siRNA concentration.[8] However, its experimental conditions were different from the current study. Peptide/siRNA complexes were incubated with the cultured cells in medium without serum in the previous report, but they were incubated with the cultured cells in medium containing serum in the current study. Peptide-based siRNA intracellular delivery was often performed

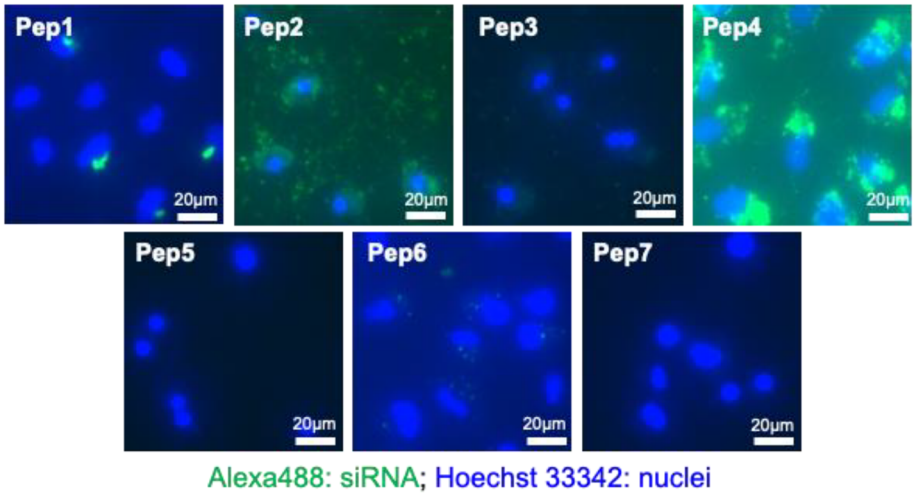


Fig. 5. Fluorescence Microscopic Observation of Huh-7-Luc Cells 24 h after Addition with **Pep1**–**Pep7**/Alexa488-siRNA Complexes at N/P = 4 and an siRNA Concentration of 100 nM

Blue: nuclei (Hoechst 33342). Green: Alexa488-siRNA.

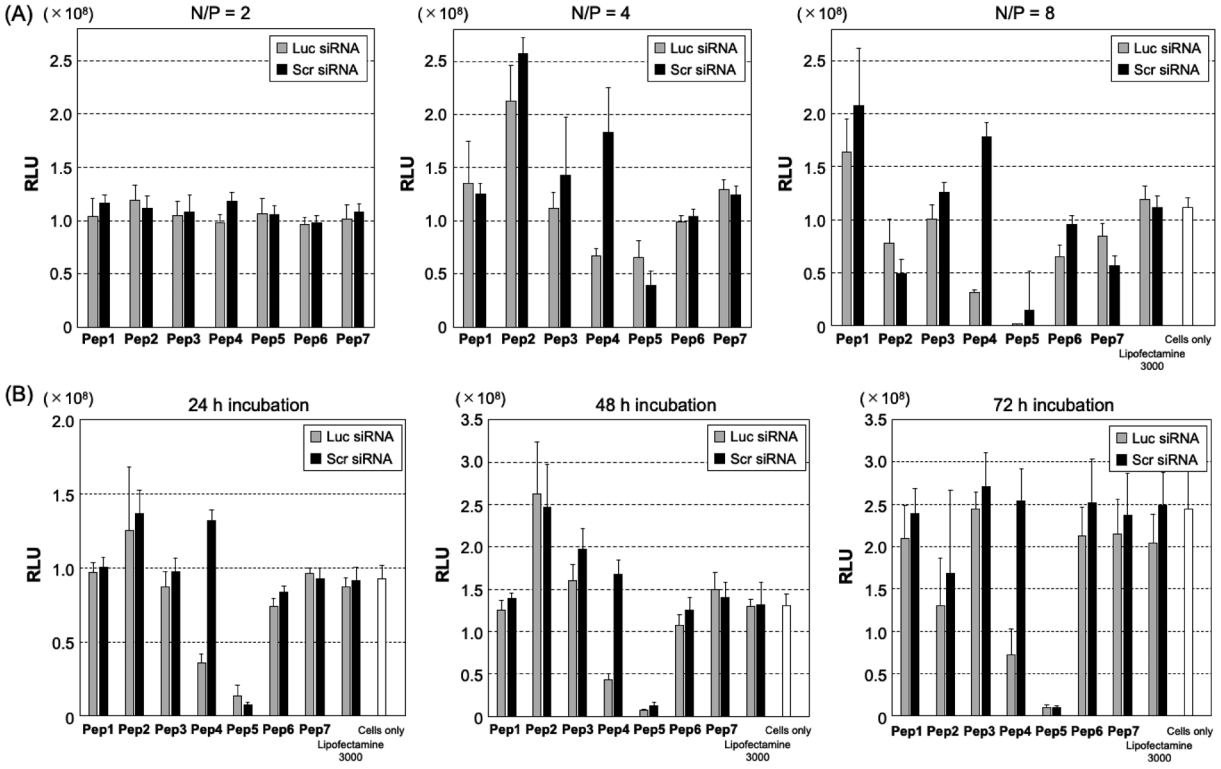


Fig. 6. Luciferase Activity of Huh-7-Luc Cells Treated with **Pep1**–**Pep7** and Lipofectamine 3000/siRNA Complexes

Gray bars: firefly GL3 luciferase siRNA (Luc siRNA). Black bars: scrambled sequence siRNA (Scr siRNA). RLU indicates relative light unit, which is the intensity of photoluminescence measured using a luciferase assay kit. Error bars represent the standard deviation ($n = 8$). Cells only indicates non-treated Huh-7-Luc cells. (A) Peptide/siRNA complexes prepared at N/P = 2, 4, and 8 were used at 100 nM siRNA with incubation for 24 h. (B) Peptide/siRNA complexes prepared at N/P = 4 were used at 100 nM siRNA with incubation for 24 h, 48 h, and 72 h.

in serum-free conditions.[12–14] Therefore, **Pep2** did not work in this study even at an siRNA concentration of 200 nM. In the meantime, the longer peptide **Pep4** produced a significant gene-silencing effect even in medium containing serum. Serum is essential for the cell growth; therefore, peptide/siRNA complexes should work in medium containing serum.

Cytotoxicity of Peptide/siRNA Complexes The cell viabilities of Huh-7 cells treated with peptide/siRNA complexes at N/P = 2, 4, and 8 were evaluated at an siRNA concentration of 100 nM after incubation for 24 h. Cationic amphipathic peptides work as a CPP, but they sometimes have a membrane-disrupting activity resulting in cytotoxicity. The results in Fig. 7 were largely consistent with those in Fig. 6A. **Pep5** exhibited severe cytotoxicity at N/P = 4 and 8. **Pep5** had two Lys, whereas other peptides except for **Pep4** (having five Lys) had four cationic amino acids Lys or Arg. In peptide/siRNA complexes at the same N/P ratios, the peptide concentration of **Pep5** was more than twice as high as that of other peptides, which might lead to higher cytotoxicity with **Pep5**. Thus, the cytotoxicities of similar amphipathic helical peptides were affected by the concentration of peptide in peptide/siRNA complexes. However, it seems to be difficult to conclude the **Pep5** cytotoxicity from only the peptide concentration. **Pep5** may have other functions that are still not clear. The cell viabilities of Huh-7-Luc cells were also decreased by treatment with **Pep2**, **Pep3**, and **Pep7**/siRNA complexes at N/P = 8. These were consistent with the results in Figs. 6 and Supplementary Fig. S2, in which the results with **Pep2**, **Pep3**, and **Pep7** implied their cytotoxicities.

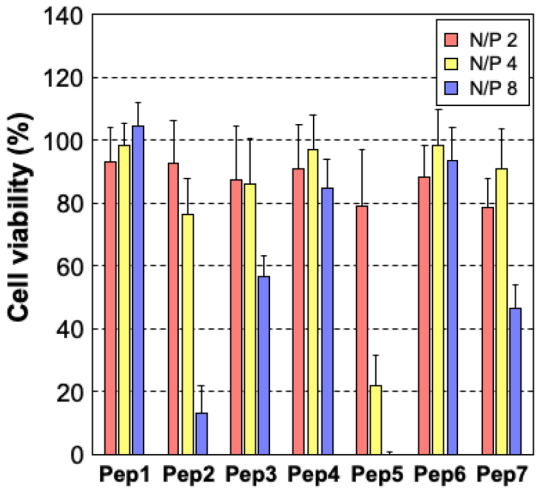


Fig. 7. Cell Viabilities of Huh-7-Luc Cells Treated with **Pep1**–**Pep7**/siRNA Complexes at Various N/P Ratios (siRNA Concentration: 100 nM; Incubation Time: 24 h)

Error bars represent the standard deviation, $n = 8$.

Conclusion

In summary, we designed seven peptides to deliver siRNAs intracellularly in medium containing serum. One of them, a Dpg-containing amphipathic helical peptide, exhibited an effective gene-silencing effect using small amounts of peptides with negligible cytotoxicity. Introduction of Dpg into peptides induced the formation of a helical structure. Peptides adopting an amphipathic structure formed complexes with siRNA with small amounts of peptides. Peptide length was critical

for siRNA intracellular delivery efficiency. These findings revealed that adequate peptide length and formation of an amphipathic structure contributed to complexation of the peptide with siRNA and effective intracellular delivery of siRNAs. The results of the current study provide a strategy for further development and design for novel CPPs to deliver siRNA intracellularly.

Experimental

Materials MALDI-TOF-MS spectra were taken on an Ultraflex (Bruker Daltonics, Fermont, CA, U.S.A.). Fmoc-protected amino acids were obtained from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). CLEAR-Amide resin was purchased from the Peptide Institute, Inc. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Phosphate-buffered saline (PBS) was obtained from Wako Chem. Co., Inc. (Osaka, Japan). Hoechst 33342 and Cell Counting Kit-8 were purchased from Dojindo Laboratories (Kumamoto, Japan). A luciferase assay kit was obtained from Promega (Madison, WI, U.S.A.). A series of siRNAs were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) and their sequences were as follows: (1) firefly GL3 luciferase (Luc siRNA): 50-CUU ACG CUG AGU ACU UCG AdTdT-30 (sense), 50-UCG AAG UAC UCA GCG UAA GdTdT-30 (antisense); (2) control scramble sequence (Scr siRNA): 50-UUC UCC GAA CGU GUC ACG UdTdT-30 (sense), 50-ACG UGA CAC GUU CGG AGA AdTdT-30 (antisense). Alexa Fluor® 488 (Alexa488) was introduced to the 50-end of the antisense strand.

Synthesis and Characterization of Peptides The peptides were synthesized on a solid support using Fmoc solid-phase methods with standard commercially available Rink amide resin and Fmoc-amino acids. The following describes a representative coupling and deprotection cycle at a 25 μmol scale. First, 59.5 mg of CLEAR-Amide resin (loading: 0.42 mmol/g) was soaked overnight in *N*,*N*-dimethylformamide (DMF). After DMF had been removed, 20% piperidine in DMF was added to the resin for deprotection. After removing and washing out piperidine, Fmoc-amino acid (4 equivalent (equiv.)) dissolved in DMF (1.0 mL), and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (4 equiv.), 1-hydroxy-7-azabenzotriazole (4 equiv.), and diisopropylethylamine (8 equiv.) dissolved in DMF (1.0 mL) were added for the coupling reaction. The resin was then suspended in cleavage cocktail (trifluoroacetic acid [TFA]: 2.8 mL; triisopropylsilane: 75 μL; ethanedithiol: 30 μL; H_2O: 75 μL). The TFA solution was evaporated to a small volume and added to cold diethyl ether (40 mL) to precipitate the peptides. The dried crude peptides were dissolved in acetonitrile and/or H_2O, and then purified by RP-HPLC using a COSMOSIL Packed Column 5C_{18}-AR-II (20 ID × 250 mm) (Nacalai Tesque, Inc., Kyoto, Japan). Freeze-drying afforded white crystals, which were characterized by analytical RP-HPLC (COSMOSIL Packed Column 5C_{18}-AR-II, 4.6 ID × 250 mm) and MALDI-TOF-MS. RP-HPLC was performed utilizing JASCO-PU-2089 Plus (JASCO, Tokyo, Japan) with a JASCO-2075-Plus as a detector. Solvent A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in acetonitrile. The purification procedure required gradient conditions (from 95 to 5% solvent A over 30 min) with a flow rate of 10 mL/min and detection at 220 nm. The purity of the final compounds was further confirmed using similar RP-HPLC conditions (from 95 to 5% solvent A over 30 min) with a flow rate of 1.0 mL/min.

CD Spectral Measurements CD spectra were measured using a JASCO CD spectrometer with 1.0 mm path length cell. Peptides were dissolved in ultrapure water at 100 μM concentration. Spectra were recorded from 250 to 190 nm.

Preparation of Peptide/siRNA Complexes Each peptide and siRNA were dissolved separately in 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 7.3). Peptide solutions of various concentrations were added to the siRNA solution to form peptide/siRNA complexes with different compositions in HEPES buffer. Complex solutions were stored at room temperature for 15 min prior to use. The N/P ratio was defined as molar ratio of the amino or guanidino groups in peptides to the phosphate groups in siRNA.

Agarose Gel Electrophoresis Gel electrophoresis was conducted at 100 V for 45 min using 4% agarose 21 gel. siRNA in the gel was stained with SYBR Green and detected using GelDoc Go Gel Imaging System (BIO-RAD Laboratories, Inc., Hercules, CA, U.S.A.).

DLS Measurements The sizes of the peptide/siRNA complexes were evaluated by DLS using Nano ZS (Malvern, Herrenberg, Germany). A He-Ne ion laser (633 nm) was used as the incident beam. Light scattering data were obtained at a detection angle of 173° and a temperature of 25 °C and were subsequently analyzed by the cumulant method to obtain the hydrodynamic diameters and PDI (μ/Γ^2) of the complexes. Results were presented as the mean and standard deviation obtained from 3 measurements.

Cellular Uptake of siRNA Huh-7-Luc cells (JCRB Cell Bank, Osaka, Japan) were seeded onto 24-well culture plates (30000 cells/well) and incubated overnight in 500 μL of DMEM containing 10% FBS. The culture media was then replaced with fresh media containing 10% FBS, and peptide/Alexa488-siRNA complex solutions at N/P = 4 were added to each well at 100 nM siRNA concentration. After incubation for 24 h, the medium was removed, and the cells were washed with ice-cold PBS, trypsinized, and collected. The fluorescence intensities of the collected cells were measured using flow cytometry RF-500 (Sysmex, Hyogo, Japan).

Fluorescence Microscopic Observation Huh-7-Luc cells were seeded onto 24-well culture plates (30000 cells/well) and incubated overnight in 500 μL of DMEM containing 10% FBS. The culture media was then replaced with fresh media containing 10% FBS, and peptide/Alexa488-siRNA complex solution at N/P = 4 was added to each well at 100 nM siRNA concentration. After incubation for 24 h, the medium was removed, and the cells were washed 3 times with ice-cold PBS. The intracellular uptake of Alexa488-siRNA was observed using a fluorescence microscope BX-X800 (Keyence, Osaka, Japan) after staining nuclei with Hoechst 33342. Observations were performed using a 20× objective lens.

RNAi Assay by Luciferase Assay Luciferase-expressing human hepatoma cell line, Huh-7-Luc cells were seeded onto 96-well culture plates (5000 cells/well) and incubated overnight in 100 μL of DMEM containing 10% FBS. The culture media were exchanged with fresh media containing 10% FBS and peptide/siRNA complex solutions were added to be at an appropriate concentration. After each incubation time, the

luciferase gene silencing was then evaluated based on the intensity of photoluminescence using a luciferase assay kit and Lumat3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Cell Viability Huh-7-Luc cells were seeded onto 96-well culture plates (5000 cells/well) and incubated overnight in 100 μL of DMEM containing 10% FBS. The culture media were then exchanged with fresh media containing 10% FBS and peptide/siRNA complex solutions were added to be at 100 nM siRNA. After incubation for 24 h, cell viabilities were evaluated using a Cell Counting Kit-8 in accordance with the manufacturer's protocol.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

References

1) Zhang M. M., Bahal R., Rasmussen P., Manautou J. E., Zhong X.-B., *Biochem. Pharmacol.*, **189**, 114432 (2021).
2) de Brito e Cunha D., Frederico A. B. T., Azamor T., Melgaco J. G., da Costa Neves P. C., Bom A. P. D. A., Tilli T. M., Messailidis S., *Pharmaceutics*, **15**, 575 (2022).
3) Oba M., *ChemBioChem*, **20**, 2041–2045 (2019).
4) Xie J., Bi Y., Zhang H., Dong S., Teng L., Lee R. J., Yang Z., *Front. Pharmacol.*, **11**, 697 (2020).
5) Stiltner J., McCandless K., Zahid M., *Pharmaceutics*, **13**, 890 (2021).
6) Staecker H., Jokovic G., Karpishchenko S., Kienle-Gogolok A., Krzyzaniak A., Lin C.-D., Navratil P., Tzvetkov V., Wright N., Meyer T., *Otol. Neurotol.*, **40**, 584–594 (2019).
7) Fortunato F., Rossi R., Falzarano M. S., Ferlini A., *J. Clin. Med.*, **10**, 820 (2021).
8) Furukawa K., Tanaka M., Oba M., *Bioorg. Med. Chem.*, **28**, 115402 (2020).
9) Oehlke J., Krause E., Wiesner B., Beyermann M., Bienert M., *Protein Pept. Lett.*, **3**, 393–398 (1996).
10) Oehlke J., Scheller A., Wiesner B., Krause E., Beyermann M., Klauschenz E., Melzig M., Bienert M., *Biochim. Biophys. Acta*, **1414**, 127–139 (1998).
11) Scheller A., Wiesner B., Melzig M., Bienert M., Oehlke J., *Eur. J. Biochem.*, **267**, 6043–6050 (2000).
12) Van Asbeck A., Beyerle A., McNeill H., Bovee-Geurts P. H. M., Lindberg S., Verdurmen W. P. R., Hällbrink M., Langel Ü., Heidenreich O., Brock R., *ACS Nano*, **7**, 3797–3807 (2013).
13) Wada S., Iwata M., Ozaki Y., Ozaki T., Hayashi J., Urata H., *Bioorg. Med. Chem.*, **24**, 4478–4485 (2016).
14) Ali S., Dussouillez C., Padilla B., Frisch B., Mason A. J., Kichler A., *J. Gene Med.*, **24**, e3401 (2022).
15) Lam J. K. W., Liang W., Lan Y., Chaudhuri P., Chow M. Y. T., Witt K., Kudsiova L., Mason A. J., *J. Control. Release*, **158**, 293–303 (2012).
16) Sajid M. I., Mandal D., El-Sayed N. S., Lohan S., Moreno J., Tiwari R. K., *Pharmaceutics*, **14**, 881 (2022).
17) Oba M., Kunitake M., Kato T., Ueda A., Tanaka M., *Bioconjug. Chem.*, **28**, 1801–1806 (2017).
18) Oba M., Nagano Y., Kato T., Tanaka M., *Sci. Rep.*, **9**, 1349 (2019).
19) Greenfield N., Fasman G. D., *Biochemistry*, **8**, 4108–4116 (1969).
20) Chang C.-T., Wu C. S. C., Yang J. T., *Anal. Biochem.*, **91**, 13–31 (1978).
21) Brahms S., Brahms J., *J. Mol. Biol.*, **138**, 149–178 (1980).
22) Forood B., Feliciano E. J., Nambiar K. P., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 838–842 (1993).
23) Oba M., *Biol. Pharm. Bull.*, **36**, 1045–1051 (2013).