This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This Accepted Manuscript will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Utilization of a PNA-peptide Conjugate to Induce a Cancer Protease-Responsive RNAi Effect

Eun Kyung Lee, Chan Woo Kim, Hiroyuki Kawanami, Akihiro Kishimura, Takuro Niidome, Takeshi Mori, and Yoshiki Katayama

Small interfering RNA (siRNA) is regarded as a promising tool for cancer therapy because of the wide applicability to various cancer-related genes. However, non-specific delivery of siRNA is one of the major causes of adverse effects. To access the issue, here we designed new siRNA system which turns on RNAi responding to a cancer cell-specific protease, cathepsin B. The system uses a peptide nucleic acid (PNA)-peptide conjugate to provide this protease-responsive activation. The PNA-peptides were found to form hybrids with double-stranded RNAs with complementary protruding regions, which then affected the susceptibility of dsRNA to Dicer. The dsRNA/PNA-peptide hybrids were activated in cancer cells with a high cathepsin B activity to show RNAi.

1. Introduction

RNA interference (RNAi) is a natural mechanism of gene silencing conserved in plant and mammalian cells. This process represents a promising new strategy for gene target identification, and has also rapidly emerged as promising therapeutic potential for human diseases including cancer. Small interfering RNAs (siRNAs), that are generally composed of 19–21 nucleotides (nt) of double-stranded (ds) RNA (dsRNA) segments with a 2-nt overhang at the 3′-end. They are produced from long dsRNA following cleavage by the Dicer enzyme. This siRNA is assembled into RNA-induced silencing complexes (RISC). The antisense strand of siRNA guides the complex to the target messenger RNA (mRNA) where mRNA degradation is initiated to inhibit protein synthesis.

The advantage of siRNA-based cancer therapy is its versatility to target a large number of different genes that are related to distinct cell signaling pathways. The major cell signaling pathways for cancer survival and proliferation can be blocked by treatment of siRNA to target cancer-related genes such as B-cell CLL/lymphoma 2,9,10 epidermal growth factor receptor,11-13 protein kinase Cα,10 vascular endothelial growth factor receptor,14 and ribonucleotide reductase subunit M2 (RRM2).15-18

Despite the advantage of siRNA-based cancer therapy, it has several issues to overcome, such as chemical instability of siRNA, off-target effects, and non-specific delivery. The non-specific delivery of siRNA to normal tissue can cause serious adverse effects because the same signaling pathways may also be essential for normal cells. To overcome non-specific delivery, researchers have used siRNAs modified with ligand molecules such as peptides,14, 21 or aptamers,22-24 which recognize cancer cell surface antigens.

An alternative way to target cancer cells with siRNA is to activate siRNA upon recognition of the specific intracellular environment of the cancer cell. Several promising strategies have been reported using this concept. Sando et al. reported an siRNA which is activated upon hybridization with a specific mRNA existing specifically in the target cell.25 Koehn et al. developed a protease-responsive siRNA by covalent modification of siRNA with a protease-specific peptide sequence.26 With the cleavage of the peptide region by caspase 4, which exists in apoptotic cells, the siRNA can be recognized by Dicer to induce RNAi. This strategy is potentially applicable to any kind of protease, although covalent modification of a peptide with siRNA is troublesome because of the chemically unstable nature of siRNA.

Here, we developed a simple protease-responsive RNAi system without covalent modification of siRNA. We designed a PNA-peptide conjugate which renders protease responsive characteristics to siRNA. The PNA-peptide conjugate is readily prepared by Fmoc-solid phase peptide synthesis.27, 28 The mechanism of action of the hybrid between PNA-peptide and dsRNA is shown in Fig. 1. To provide the protease responsive action, dsRNA that composes of normal antisense RNA (asRNA) and sense strand RNA (ssRNA) with a protrusion at 3′-terminus, was also designed and synthesized.
The PNA region of the PNA-peptide (gray sequence) hybridizes with ssRNAs protruded.

(a) Mechanism of action of cancer protease-responsive siRNA.

(b) RNA Sequences

ssRNA-S: 5′-CUUACGCUAGAUUCAGACGAdTdT-3′
ssRNA-L: 5′-CUUACGCUAGAUUCAGACGAdTdT-3′
asRNA: 5′-GAGUAGAGAGAGAGAGAGAGGAdTdT-3′

(c) PNA-peptide Sequences

r8S: NH₂-rmm-GLF-X-CTACTACTA-H₂O
r8S-N: NH₂-rmm-GLF-X-CTACTACTA-H₂O
r4S: NH₂-rarahara-GLF-X-CTACTACTA-H₂O
r4S-N: NH₂-rarahara-GLF-X-CTACTACTA-H₂O
r4L: NH₂-rarahara-X-CTACTACTA-H₂O
r4L-N: NH₂-rarahara-X-CTACTACTA-H₂O

Fig. 1 (a) Mechanism of action of cancer protease-responsive siRNA. Sequences of RNA (b) and PNA-peptide (c) used here. (X: 8-amino-3,6-dioxoanatoic acid). Sequences shown in gray and green in PNA-peptide are sterically-hindering region and complementary region, respectively. The lowercase letters indicate D-amino acids.

2. Experimental section

2.1 Materials

NovaSyn TGR resin and all 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids were purchased from Novabiochem, Merck (Tokyo, Japan). Peptide nucleic acids (PNA) were purchased from PANAGENE (Daejeon, Korea). 1-hydroxybenzotriazole monohydrate (HOBT·H₂O), O-benzotriazol-1-·N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,N'-diisopropylethylamine (DIEA), piperidine (PD), and trifluoro acetic acid (TFA) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). N,N-dimethylformamide (DMF) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Trisopropylsilane (TIS) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Triethylamine (TEA), 250 µL/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and Opti-MEM were purchased from Gibco Life Technologies (Grand island, NY, USA). Tris-Borate-EDTA (TBE) buffer and TritionX-100 were purchased from Gibco Life Technologies (Grand island, NY, USA). Human liver cathespin B was purchased from MBL (Nagoya, Japan). siRNA ladder was purchased from Takara biotechnology (Tokyo, Japan). Recombinant Dicer Enzyme Kit was purchased from Genlantis, Inc. (San Diego, CA, USA). Lipofectamine™2000 reagent was purchased from Invitrogen (Carlsbad, California, USA). Luciferase assay substrate was purchased from Promega (Madison, WI, USA). Luciferase (Luc) siRNA and double strand (ds) RNA were purchased from BONAC Corporation (Kurume, Japan) with the following sequence: Luc sense strand: 5′-CUUACGCUAGAUUCGAdTdT-3′, Luc antisense strand: 5′-UCGAGAUCUAACGCGUAAdTdT-3′, ds sense strand: 5′-CUUACGCUAGAUUCGAdTdT-3′, and ds antisense strand: 5′-GACAUUUCGAAGACUCAGCACGUAAdGAdA-3′

2.2 Synthesis of PNA-peptide

All of PNA-peptides were prepared by standard Fmoc-solid phase peptide synthesis using NovaSyn TGR resin (0.25 mmol/g). After Fmoc deprotection using 20% piperidine in DMF for 10 min, Fmoc-protected amino acid (3 eq.) or PNA (1.5 eq.) were coupled by HOBt/HBTU (3 eq.) and DIEA (6 eq.) in DMF. For cleaving protection groups, TFA/water/triisopropylsilane (95/2.5/2.5) mixture was added to the resin and placed for 90 min with shaking. Then, PNA-peptide in the resulting solution was collected by reprecipitation against 40 mL of cold diethyl ether. The crude PNA-peptides were purified by a LaChrome Elite reverse-phase liquid chromatography (Hitachi High-Technologies Corporation, Tokyo, Japan). All of the PNA-peptides were eluted with a gradient of 0-30% eluent A and B within 30 min at a flow rate of 1.0 ml/min, where A was 0.1% (v/v) TFA in water and B was 0.1% (v/v) TFA in acetonitrile on a Atlantis dC18 reversed phase column (5 µm, 4.6×100 mm). Detection and purity were performed and determined at 260 nm. The molecular weight of PNA-peptide was identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Bruker autoflex-III spectrometer using cyano-4-hydroxybenzoinic acid (CHCA) as matrix. m/z: r8S calcd for C₁₇H₁₁₂N₈O₄S₄ 4307.43; found 4306.52, r8S-N calcd for C₁₆H₁₁₁N₈O₄S₄ 4078.27; found 4079.20, r4S calcd for C₁₃H₈₂N₂₇O₄S₄ 3967.22; found 3967.96, r4S-N calcd for C₁₃H₈₂N₂₇O₄S₄ 3738.06; found 3740.66, r4L calcd for C₁₃H₈₂N₂₇O₄S₄ 4484.50; found 4485.34, r4L-N calcd for C₁₃H₈₂N₂₇O₄S₄ 4257.35; found 4258.20.

2.3 Concentration determination of PNA-peptide

Concentration of PNA-peptide are calculated by each base extinction coefficient at 260 nm (ε_PNA,A = 17300 M⁻¹ cm⁻¹, ε_PNA,G = 17100 M⁻¹ cm⁻¹, ε_PNA,C = 6600 M⁻¹ cm⁻¹, ε_PNA,T = 8600 M⁻¹ cm⁻¹).

2.4 Cleavage assay of PNA-peptide by Cathepsin B

PNA-peptides were dissolved in reaction buffer (50 mM NaOAc, 1 mM EDTA, 1 M DT, pH 5.4) to be a concentration of 20 µM and then 5.9 U/ml human liver Cathepsin B (MBL, Nagoya, Japan) was added to the solution and incubated a 37 oC for 30 min. After 30 min, cleavage site of PNA-peptide were diluted with 10 µg/mL CHCA solution and was analyzed by MALDI-TOF mass spectrometry.
2.5 Melting temperature of PNA-peptide with dsRNA
Solution of PNA-peptide and dsRNA were all brought to 3 µM. The duplex were formed by annealing at 90 °C for 1 min and then gradually cooled to 5 °C. Heating rate of the melting measurement was 1 °C/min.

2.6 Formation of dsRNA/PNA-peptide hybrid
dsRNA and PNA-peptides (3 µM) in PBS were heated to 90 °C for 1 min for annealing, then gradually cooled to room. The resulting dsRNA/PNA-peptide hybrid was electrophoresed in a 40% polyacrylamide gel in TBE buffer (Sigma Aldrich, St. Louis, MO, USA). During the electrophoresis, the gel temperature was ice-cooled to avoid the dissociation of the hybrid. RNAs and hybrids were visualized by soaking the gel in distilled water containing SYBR Gold nucleic acid gel stain (Molecular Probe, Inc., Eugene, Oreg.).

2.7 Dicer processing
dsRNA/PNA-peptide (8 µM) hybrid was diced using a 1 U/mL of Recombinant Dicer Enzyme Kit (Gene Therapy Systems, Inc., San Diego, CA, USA) in reaction buffer at 37 °C for 20 h (total 10 µL). After the reaction, reaction was quenched by adding 2 µL of stop solution to the reaction mixture. The resulting solution was analyzed by 40% polyacrylamide gel electrophoresis in TBE buffer under ice-cooling. RNAs and hybrids were visualized by soaking the gel in distilled water containing SYBR Gold nucleic acid gel stain (Molecular Probe, Inc., Eugene, Oreg.).

2.8 Cell culture
CT-26 stably expressing firefly luciferase was kindly provided by Dr. Atsushi Maruyama (Tokyo Institute of Technology) and Dr. Ario Kano (Kyushu University). CT-26 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all from Giboco Life Technologies, Grand island, NY, USA) in humidified atmosphere with 5% CO₂ and 95% air at 37 °C.

2.9 Cytotoxicity of PNA-peptide
CT-26 cells were seeded in 96-well plates (1×10⁵ cells/well) at 37 °C in DMEM before the assay. After 24 h incubation, PNA-peptide (0 to 50 nM) solution containing Lipofectamine 2000 was added in each well. After 4 h, medium containing 10% FBS was replaced and incubated for 20 h. A 10 µL of Cell Counting Kit-8 regent (Dojindo Laboratories, Kumamoto, Japan) were added to each well and were incubated for 2 h. Then, 96-well plate was measured at 450 nm by microplate reader.

2.10 Lysosomal cathepsin B activity
To monitor cathepsin B activity, CT-26 cells were seeded in 96-well glass plates (1×10⁴ cells/well) until 80% confluent. After 24 h, CT-26 cells were incubated for 3 h with 0 to 10 µM cathepsin B inhibitor (CA-074 Me, L-trans-Epoxysuccinyl-Lle-Pro-OMe propylamide, Bachem AG, Bubendorf, Switzerland). Then, Magic red cathepsin B substrate reagent solution (MR-(RR)₂, Immuno Chemistry Technologies, Bloomington, MN, USA) was added to each medium at a volume ratio of 1:26, and incubated for 1 h at 37 °C. Then, the medium was removed and the cells were rinsed with PBS. The cells were stained with Hoechst 33342 for 10 min and analyzed by Biozero fluorescence microscope (BZ-8000, Keyence, Osaka, Japan).

2.11 Transfection
CT-26 cells were seeded in 24-well plates (2×10⁴ cells/well) at 37 °C in DMEM containing 10% FBS and grown to 30-50% confluence. For the inhibition of cathepsin B activity, the cells were washed with PBS and then incubated for 3 h at 37 °C in serum-free medium with cathepsin B inhibitor (CA-074 Me, 0 to 10 µM). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA). Before transfection, 20 nM siRNA or 20 nM dsRNA/PNA-peptide hybrid was added respectively in Opti-MEM (Giboco) and Lipofectamine 2000 was diluted with Opti-MEM. Each solution was incubated for 5 min at room temperature. Then, they were combined and mixed gently and then incubated for 20 min at room temperature. When solution appeared cloudy after 20 min, the solutions were added to each well containing cells and serum-free medium. After 6 h incubation, medium was replaced to DMEM, and the cells were cultured for 42 h. To investigate the RNAi effect, the cells were lysed with 200 µL of lysis buffer [20 mM Tris-HCl, 0.05% TritonX-100, 2 mM EDTA (pH 7.5)] for 20min. A 10-µL of the lysate solution was mixed with 40-µL of luciferase assay solution (Promega, Madison, WI, USA) and then measured with a luminometer (GloMax 20/20n, Promega, Madison, WI, USA).

3. Results and discussion
PNA-peptides summarized in Fig. 1c were synthesized by Fmoc-solid phase peptide synthesis. PNA-peptides were purified by reverse phase HPLC and were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. D-amino acid was used so that the sterically-hindering region was resistant to non-specific proteolytic degradation (shown as lowercase letters in Fig. 1c). The PNA-peptide includes a substrate sequence for cathepsin B (GFLG) in r8S, r4S and r4L, and as lowercase letters in Fig. 1c). The peaks detected in a mass spectrum of original r8S (Fig. S1). We checked whether the PNA-peptides can be cleaved by cathepsin B. The PNA-peptides were dissolved in reaction buffer and cathepsin B was added to the solution. After incubation of PNA-peptide with cathepsin B, the reaction mixture was analyzed by MALDI-TOF mass spectrometry. As shown in Fig. 2a, r8S was cleaved selectively at the various positions of the GFLG sequence. In contrast, a negative control r8S-N, which does not include this sequence, was not cleaved by cathepsin B (Fig. 2b). The peaks observed in r8S-N are assignable to impurities which were also detected in a mass spectrum of original r8S (Fig. S1).

We examined solubility of PNA-peptides and their hybrids with complementary RNA in physiological saline by turbidimetry (Fig. S2). When r8S was mixed with dsRNA-S (ssRNA-S/asRNA duplex), the solution became slightly turbid. This insolubility of the hybrid could result from the strong electrostatic interaction between
anionic dsRNA-S and cationic r8S. Then we examined r4S, which contained half the number of cationic D-arginine (Fig. 1b). Although r4S was not completely soluble in PBS due to the less cationic charge and hydrophobic nature of PNA, it became soluble upon hybrid formation with dsRNA-S (Fig. S2). Then we measured the melting curve of this hybrid. As shown in Fig. 3, dsRNA-S/r4S hybrid (blue line) showed two transitions at 40°C and 70°C. The higher Tm was found to be the melting temperature of dsRNA-S (Fig. S3). Thus, the lower Tm was attributed to the melting point of dsRNA-S and r4S. The Tm between dsRNA-S and r4S was not suited to further experiments because the hybrid would be unstable at physiological temperature, 37°C. Thus, we designed another PNA-peptide, r4L (Fig. 1c), that was extended by two bases in the PNA region of r4S to stabilize the hybrid with dsRNA-L (ssRNA-L/asRNA duplex). The dsRNA-L/r4L hybrid was fully soluble in PBS (Fig. S2) and like the dsRNA-S/r4S hybrid (Fig. S3). Thus, we selected r4L for RNAi applications as shown below.

Fig. 2 MALDI-TOF mass spectra of cathepsin B digested fragments from r8S (a) and r8S-N (b).

**Fig. 3** Melting behavior of dsRNA-S/r4S hybrid (blue line) and dsRNA-L/r4L hybrid (red line) in PBS. Arrows indicate melting temperatures. Concentration of each hybrid is 3 μM. Heating rate is 1.0°C/min.

showed two transitions (Fig. 3, red line). From the melting curve, the Tm value between dsRNA-L and r4L was determined to be 52°C, which is much higher than physiological temperature. Thus, we selected r4L for RNAi applications as shown below.

The formation of dsRNA-L/r4L hybrid was confirmed by polyacrylamide gel electrophoresis (PAGE). After annealing r4L and RNA, the mixture was applied to PAGE. As shown in Fig. 4, dsRNA-L/r4L hybrid showed a single band with no remaining fluorescence in the well, indicating the quantitative formation of the hybrid and complete dissolution of the hybrid in aqueous medium. The lower mobility of the hybrid compared with dsRNA-L could be explained by an increase in molecular weight and addition of D-arginine’s cationic charge following hybridization with r4L.

Then we examined resistance of dsRNA/r4L hybrid to Dicer as conceptually depicted in Fig. 1. After treating dsRNA/r4L hybrid with recombinant human Dicer, the resulting mixture was analyzed by PAGE. As shown in Fig. 5, in the case of dsRNA, a new band resulting from the blunt-ended dsRNA created by Dicer appeared (lane 2). However, the dsRNA/r4L hybrid did not provide the blunt-ended dsRNA and seemed to be completely intact (lane 4). This indicated that the peptide region of PNA-peptide hinders Dicer’s nuclease activity.
Before examining whether RNAi occurred, we checked the cytotoxicity of the PNA-peptide, r4L, on CT-26 cells (Fig. S2). The cytotoxicity was almost negligible when the r4L concentration was less than 20 nM. Therefore, we used the PNA-peptide at 20 nM in subsequent RNAi experiments.

We evaluated RNAi of dsRNA-L/r4L hybrid in CT-26 cells that constitutively expressed firefly luciferase. CT-26 cells have been reported to highly express cathepsin B. Lipofectamine 2000 was used to transfect the hybrid into the cells because we found that the cellular uptake of the hybrid alone was negligible. RNAi effect was evaluated 48 hours after addition of the hybrid. As shown in Fig. 6, the dsRNA-L/r4L hybrid reduced the luciferase activity to ca.20%, which is similar to that of siRNA and dsRNA alone. In contrast, the negative control, dsRNA-L/r4L-N hybrid, which does not include a cathepsin B cleavable site shows high level of luciferase activity (>60%) compared with the dsRNA-L/r4L hybrid. Thus, the significant difference of luciferase activity between these two hybrids should result from the activation of RNAi by the dsRNA-L/r4L hybrid that corresponded to cathepsin B activity as depicted in Fig. 1a. An incomplete suppression of the RNAi effect of the negative control dsRNA-L/r4L-N hybrid may be due to the dissociation of some amount of the hybrid in intracellular conditions to generate free dsRNA.

To further obtain the evidence of the cathepsin B-responsive RNAi effect, we examined the effect of cathepsin B inhibitor on the RNAi effect of the hybrid. First we confirmed the effect of a cathepsin B inhibitor (CA-074 Me) by using commercial cathepsin B fluorescent probe, Magic red cathepsin B substrate, which becomes fluorescence after hydrolysis by cathepsin B in lysosome. As shown in Fig. 7, untreated cells showed a strong red fluorescence in the cytosol resulting from the cathepsin B probe. However, the red fluorescence became very weak in the presence of the inhibitor (10 µM). Thus, cathepsin B activity can be inhibited by CA-074 Me in CT-26 cells. Then we examined the inhibitory effect of cathepsin B on the RNAi effect of the hybrid. As shown in Fig. 8, the suppression of the luciferase activity by dsRNA-L/r4L hybrid was weakened with increasing concentration of the inhibitor. At the highest concentration of the inhibitor (10 µM), the luciferase activity recovered to almost same level with the negative control, dsRNA-L/r4L-N. These results are another evidence that the RNAi effect of dsRNA-L/r4L hybrid was triggered by the cellular cathepsin B as depicted in Fig. 1a.

Conclusions

In conclusion, we successfully designed PNA-peptides which were expected to regulate the RNAi activity in response to the cancer-specific protease cathepsin B. The PNA-peptide was found to be cleavable by cathepsin B and stably formed a hybrid with dsRNA that has a protruding 3′ region for hybridization. This hybrid successfully showed the RNAi effect in a cancer cell line with high cathepsin B activity. The hybrid lacking the cathepsin B cleavage site showed a minimal RNAi effect. Our design of the hybrid is universally applicable to any kind of protease.

Acknowledgements

We thank Dr. Atsushi Maruyama (Tokyo Institute of Technology) and Dr. Arihiro Kano (Kyushu Univ.) for kindly providing the firefly luciferase stably transfected CT-26 cell line. We thank Dr. Shinsuke Sando (Univ. of Tokyo) for valuable discussion.
This work was financially supported by a Grant-in-aid for Scientific Research from MEXT.

References

23. X. Li, Q. Zhao and L. Qiu, J. Controlled Release, 2013, 171, 152-162.