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SiRNAs conjugated with aromatic compounds induce RISC-mediated antisense strand selection and strong gene-silencing activity

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ABSTRACT

Short interference RNA (siRNA) is a powerful tool for suppressing gene expression in mammalian cells. In this study, we focused on the development of siRNAs conjugated with aromatic compounds in order to improve the potency of RNAi and thus to overcome several problems with siRNAs, such as cellular delivery and nuclease stability. The siRNAs conjugated with phenyl, hydroxyphenyl, naphthyl, and pyrenyl derivatives showed strong resistance to nuclease degradation, and were thermodynamically stable compared with unmodified siRNA. A high level of membrane permeability in HeLa cells was also observed. Moreover, these siRNAs exhibited enhanced RNAi efficacy, which exceeded that of locked nucleic acid (LNA)-modified siRNAs, against exogenous *Renilla* luciferase in HeLa cells. In particular, abundant cytoplasmic localization and strong gene-silencing efficacy were found in the siRNAs conjugated with phenyl and hydroxyphenyl derivatives. The novel siRNAs conjugated with aromatic compounds are promising candidates for a new generation of modified siRNAs that can solve many of the problems associated with RNAi technology.

1. Introduction

RNA interference (RNAi) technology has attracted particular attention as the most powerful means of suppressing gene expression in mammalian cells [1]. In RNAi technology, chemically synthesized short interfering RNAs (siRNAs), consisting of a 19-nucleotide (nt) duplex with a 2-nt 3′ overhang on either end, are widely used [2]. These siRNAs bind to a protein complex called an RNA-induced silencing complex (RISC) [3], resulting in cleavage of the target mRNA guided by the antisense strands of the siRNAs. However, the known problems with the siRNAs, such as poor membrane permeability, nuclease resistance, and off-target effects, should be resolved before medical and clinical application. Many kinds of chemically modified siRNAs have been developed to address these problems. Locked nucleic acid (LNA), which is a nucleic acid analog containing a 2′-O, 4′-C methylene bridge, modified siRNAs (siLNAs) have been shown to enhance thermodynamic stability and high nuclease resistance [4]. The 2′-modifications of siRNAs (e.g., 2′-O-methyl and 2′-fluoro) and modifications of a phosphate backbone (e.g., phosphorothioate and boranophosphate) demonstrated high nuclease resistance [5–9]. A series of nucleobase-modified siRNAs were reported to enhance thermodynamic stability [10–13]. Direct conjugation of polymers [14,15], peptides [16–18], and lipids [19–22] to siRNAs has been shown to mediate siRNA uptake in cells. Although the chemical modifications and direct conjugations to the siRNAs could solve some of the problems with RNAi, such as nuclease stability or cell membrane permeability, most of them substantially weaken gene-silencing efficacy.

In the present study, we designed siRNAs conjugated with four types of aromatic compounds (phenyl, hydroxyphenyl, naphthyl, and pyrenyl derivatives) to improve their biological properties, including stability against nuclease degradation, membrane permeability, and RNAi efficacy. As a control, not only unmodified siRNA but also the siLNAs were used. All siRNAs conjugated with aromatic compounds (Ar-siRNAs) showed significantly improved stability in serum, cellular uptake, and RNAi efficacy.

2. Materials and methods

2.1. Design and synthesis of siRNAs conjugated with aromatic compounds

We designed 21-nt siRNAs to target the *Renilla* luciferase genes. The siRNA sequences were as follows: *Renilla* sense, 5′-GGCCUUU-CACUACUCCUACGA-3′; *Renilla* antisense, 5′-GUAGGAGUAGUGAA

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AGGCCAG-3′. All single-strand RNAs (ssRNAs: antisense, sense, amino modifications), except for LNA modifications, were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The LNA-modified sense RNAs were purchased from Gene Design (Osaka, Japan).

The ssRNAs (sense) were conjugated with phenyl, hydroxylphenyl, naphthyl, and pyrenyl derivatives via an amino linker (Fig. 1A). The amino-modified ssRNAs (4 nmol in 20 μl water) were reacted with 40 nmol of either phenoxyacetic acid *N*-hydroxysuccinimide ester (Sigma–Aldrich, St. Louis, MO), 3-(4-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester (Sigma–Aldrich), 2-naphthoxyacetic acid *N*-hydroxysuccinimide ester (Sigma–Aldrich), or 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (Sigma–Aldrich) dissolved in 10 μl *N*,*N*-dimethylformamide (DMF, Sigma–Aldrich) containing 0.7 μl *N*,*N*-diisopropylethylamine (DIEA; Sigma–Aldrich) in 100 μl isopropanol/water (1:1) mixture solutions, for 12 h at room temperature. The ssRNAs conjugated with phenyl, hydroxylphenyl, naphthyl, or pyrenyl derivatives (Ph-ssRNA, HyPh-ssRNA, Nap-ssRNA, or Pyr-ssRNA) were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using an ODS column (4.6 × 150 mm, 5 μm) under a linear condition of acetonitrile, with the concentrations shifting from 7% to 70% during 40 min in 20 mM triethylammoniumacetate (TEAA) (pH 7.0). The molecular weights of all ssRNAs, including aromatic conjugates and LNA modifications, were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany) using saturated solutions of 2,4,6-trihydroxyacetophenone (Sigma–Aldrich) in 50 mg/ml diammonium hydrogen citrate in 50% acetonitrile as a matrix (Wako, Osaka, Japan) [23]. The concentrations of all ssRNAs were calculated using their absorbance at 260 nm detected spectrophotometrically (V-670 spectrophotometer; JASCO, Tokyo, Japan). The sense RNA strands, including aromatic conjugates and LNA modifications, were annealed with antisense RNA strands that were complementary to mRNA strands of the target *Renilla* luciferase gene in annealing buffer to prepare unmodified siRNAs, Ar-siRNAs, in which the phenyl, hydroxylphenyl, naphthyl, or pyrenyl derivative was covalently conjugated to the siRNAs at the 5′-end of the sense strand (Ph-siRNA, HyPh-siRNA, Nap-siRNA, or Pyr-siRNA) and siLNAs, in which the siRNA was modified with LNA at the 5′-end or both the 5′- and 3′-end of the sense strands (siLNA1 or siLNA2) (Fig. 1A). The quality of the Ar-siRNAs was confirmed by 20% polyacrylamide gel electrophoresis (PAGE).

2.2. Determination of melting temperature

The melting temperatures (T_m) of the siRNAs, including Ar-siRNAs and siLNAs (200 pmol), were recorded with a V-630BIO UV/Vis spectrophotometer (JASCO) in 10 μl buffer (100 mM NaCl, 20 mM Tris–HCl, pH 7.5). Absorbance at 260 nm was measured as a function of temperature from 20 to 95 °C with a 0.5 °C increase per min.

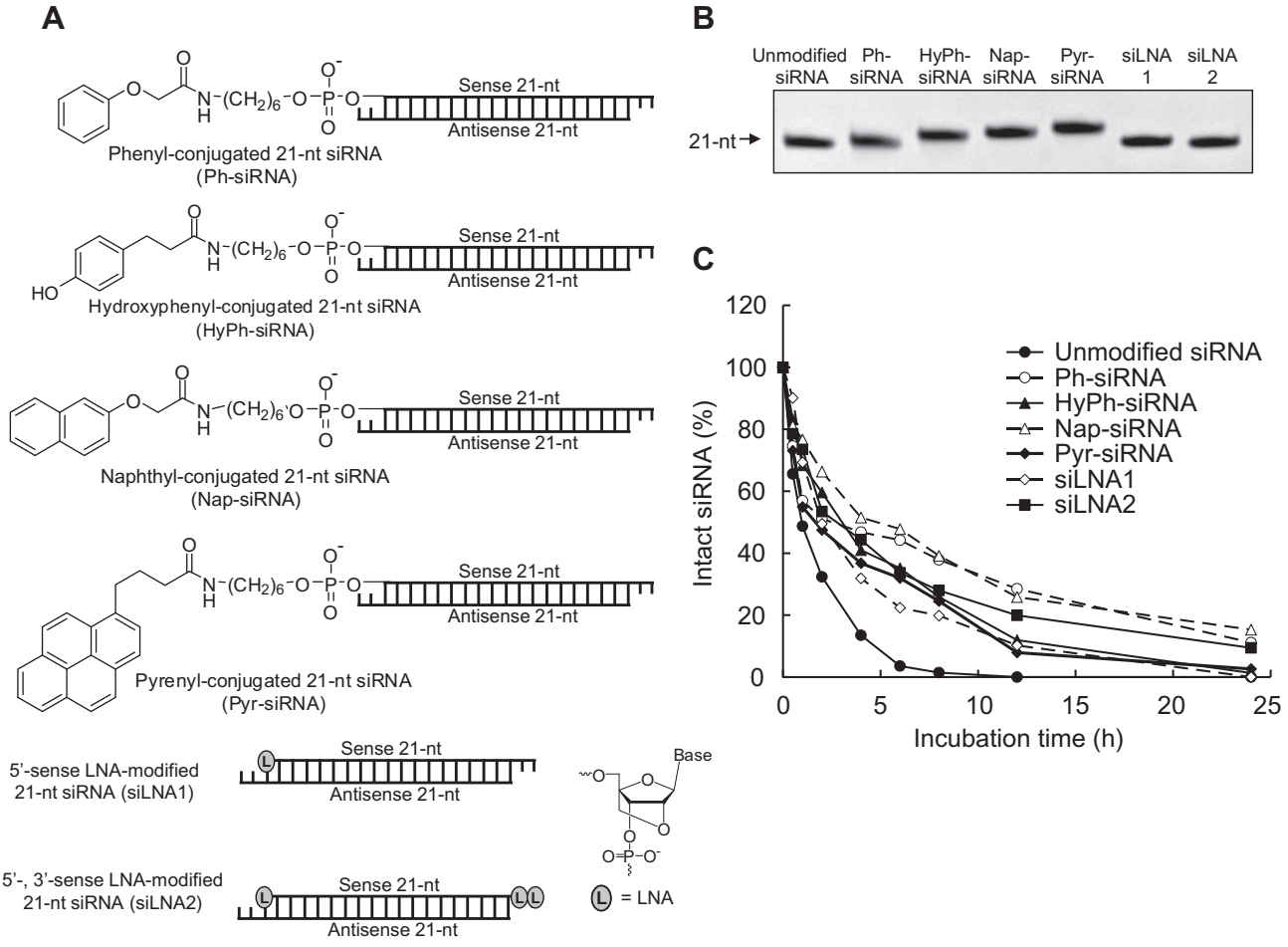

Fig. 1. (A) Structures of Ar-siRNAs and siLNAs. (B) PAGE analysis of unmodified siRNA, Ar-siRNAs, and siLNAs. (C) Survival rates of unmodified siRNA, Ar-siRNAs, and siLNAs in culture medium. Samples (unmodified siRNA, Ar-siRNAs, and siLNAs) were incubated in culture medium containing 10% FBS at 37 °C for 0, 0.5, 1, 2, 4, 6, 8, 12, or 24 h, and aliquots were analyzed using 20% PAGE. Survival rates were calculated by comparing the band intensity of each sample with that of control.

T_m values were obtained from the maxima of the first derivatives of the melting curves.

2.3. Stability against nuclease degradation in cell-cultured medium

Ten microliters of each siRNAs (200 pmol), including Ar-siRNAs and siLNAs, was added to 90 μl of Dulbecco's modified Eagle's medium (DMEM; Wako) containing 10% heat-inactivated FBS (Invitrogen, La Jolla, CA). The samples were incubated for different time periods (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h) at 37 °C. Aliquots (10 μl) were taken from each sample. The samples were frozen in liquid nitrogen (to stop the nuclease reaction) and were kept at −20 °C. The RNA products of nuclease degradation were analyzed using 20% PAGE (30 mA, 70 min) and visualized by silver staining (DNA Silver Stain Kit, GE Healthcare). The signals of the RNA products were photographed by LAS4000 (Fujifilm, Tokyo, Japan).

2.4. Cell culture and transfection

HeLa cells were cultured in DMEM (Wako) supplemented with 10% heat-inactivated FBS (Invitrogen), 100 U/ml penicillin (Wako), and 100 μg/ml streptomycin (Wako). To evaluate the RNAi potency of siRNAs, including Ar-siRNAs and siLNAs, targeting the *Renilla* luciferase gene, we used the psiCHECK-2 Vector (Promega, Madison, WI) as a reporter gene, which contains both the *Firefly* and *Renilla* luciferase genes, in HeLa cells. HeLa cells were seeded at 5×10^4 cells/ml in 100 μl medium in each well of a 96-well multiplate and cultured under a 100% humidified atmosphere (5% CO_2, 37 °C). Twelve hours later, 0.02 μg of psiCHECK-2 Vector, which was mixed with 0.2 μl of Lipofectamine 2000 (LF2000; Invitrogen) in 10 μl of Opti-MEM (Invitrogen), was added to each well containing the cells in 90 μl fresh culture medium without antibiotics. To investigate RNAi efficacy, the siRNAs, including Ar-siRNAs and siLNAs, at different concentrations (0.5, 1, 2, and 5 nM) were pre-incubated with 0.2 μl of LF2000 in 10 μl of Opti-MEM. Four hours after the vector transfection, 10 μl of the pre-incubated mixtures of siRNAs, including Ar-siRNAs and siLNAs, with LF2000 were added to each well containing 90 μl of fresh culture medium. After an another 8 h incubation, the culture medium was replaced with 100 μl fresh medium and the cells were cultured for 48 h to assess RNAi.

2.5. Gene-silencing of Renilla luciferase

The efficacy of RNAi against *Renilla* luciferase was evaluated by the Dual-Glo Luciferase Assay System (Promega). To detect *Firefly* luciferase activity as an intraplasmid control, 50 μl of Dual-Glo Luciferase reagent-1 (beetle luciferin) was added to each well of a 96-well multiplate containing siRNAs. A plate was incubated in the dark for 10 min at room temperature. Luminescence emitted from the *Firefly* luciferase catalytic reaction was measured for 1 s for each well on a microplate reader (Wallac 1420 ARVO MX; Perkin Elmer, Waltham, MA). To measure the *Renilla* luciferase activity and to quench the luminescence from the *Firefly* luciferase catalytic reaction, 50 μl of Dual-Glo Stop and Glo reagent-2 (containing coelenterazine) was added to each well. The multiplates were then incubated in the dark for 10 min at room temperature. The luminescence arising from the *Renilla* luciferase catalytic reaction was measured in the same way as described above for *Firefly* luciferase activity, and was normalized by the luminescence of *Firefly* luciferase activity in each well of the 96-well multiplates. The RNAi efficacy of unmodified siRNA, Ar-siRNAs, and siLNAs towards the *Renilla* luciferase was assessed as a percentage of the control (siRNA nontreated) sample.

2.6. Confocal laser scanning microscopy

The fluorescein-labeled antisense ssRNA (FAM-ssRNA) was purchased from IDT. The FAM-ssRNA was annealed with sense ssRNA, Ph-ssRNA, HyPh-ssRNA, Nap-ssRNA, and Pyr-ssRNA, respectively, in annealing buffer to prepare fluorescein-labeled siRNAs (FAM-siRNAs).

To deliver the prepared siRNAs intracellularly, 100 pmol of FAM-siRNAs, including Ar-siRNAs, was incubated with 2 μl LF2000 in 100 μl Opti-MEM diluted twice for 30 min at room temperature. Then, 100 μl of each sample was added to 900 μl culture medium of HeLa cells (5×10^4 cells) and incubated for 6 h in the dark under a humidified atmosphere (5% CO_2, 37 °C). The cells were washed several times with PBS (−), fixed with 4% paraformaldehyde/PBS for 15 min, and stained with Mito Tracker Red CMXRos (Molecular Probes, Eugene, OR) to visualize the mitochondria. The intracellularly incorporated amount of FAM-siRNAs in cells was examined under a confocal fluorescent microscope (IX70; Olympus, Tokyo, Japan). For quantitative assessment of fluorescence images, the images were analyzed using Scion image software (Scion, Frederick, MD).

3. Results

3.1. Synthesis of siRNAs conjugated with aromatic compounds

We selected an exogenous *Renilla* luciferase as a target gene. The sense ssRNA modified with amine at the 5′-end was condensed with aromatic compounds such as phenyl, hydroxyphenyl, naphthyl, and pyrenyl derivatives, which were activated by *N*-hydroxysuccinimide ester. The properties of ssRNAs conjugated with aromatic compounds are shown in Table 1. Overall yields for conjugated ssRNAs (Ph-ssRNA, HyPh-ssRNA, Nap-ssRNA, and Pyr-ssRNA) were 86.3%, 75.1%, 64.1%, and 42.4%, respectively, after RP-HPLC purification. The RP-HPLC retention time of each conjugate differed from that of the unmodified ssRNA under purification conditions (Fig. S1). The molecular weight of each conjugate was confirmed by MALDI-TOF mass spectrometry (Fig. S2).

Each conjugate was annealed with the antisense ssRNA, which was complementary to the mRNA strands of the target *Renilla* luciferase gene, in order to prepare Ar-siRNAs (Ph-siRNA, HyPh-siRNA, Nap-siRNA, and Pyr-siRNA). Each Ar-siRNA was confirmed to be a duplex stranded by using 20% PAGE (Fig. 1B). The Ar-siRNAs were obtained in high purity, and each corresponding band showed a different mobility from that of unmodified siRNA. The thermodynamic stability of each purified Ar-siRNA was investigated (Fig. S3). The T_m value of each Ar-siRNA is shown in Table 1. Ar-siRNAs showed slightly greater thermodynamic stability than the unmodified siRNA. The thermodynamic stability of each siLNA was also investigated, revealing slightly increased T_m values compared with that of unmodified siRNA (Table 1 and Fig. S3).

3.2. Nuclease resistance of Ar-siRNAs

The nuclease stability of siRNAs, including Ar-siRNAs and siLNAs, in culture medium containing 10% FBS was evaluated using PAGE analysis (Fig. 1C). The degradation of the unmodified siRNA began immediately, leading to the complete disappearance of the siRNA after 7 h incubation. In contrast, Ar-siRNAs exhibited greatly increased resistance against nuclease degradation. It was interesting that the Ar-siRNAs had the same levels of nuclease resistance as the siLNAs; the latter are known to have high nuclease resistance [4].

3.3. RNAi efficacy of Ar-siRNAs

We performed a gene-silencing study of siRNAs, including Ar-siRNAs and siLNAs, targeted to the *Renilla* luciferase gene. *Renilla*

Table 1
Characterizations of ssRNA conjugated with aromatic compounds.

Name	Target gene	Conjugated molecule	HPLC retention time[a] (min)	MALDI-TOF MS[b] Found/Calcd	Yield[c] (%)	T_m[d]
Unmodified ssRNA	Luciferase	None	12.7	6569.8/6569.9	–	82.40
Ph-ssRNA	Luciferase	Phenyl	17.9	6881.1/6882.1	86.3	83.50
HyPh-ssRNA	Luciferase	Hydroxyl phenyl	16.0	6895.9/6896.2	75.1	83.10
Nap-ssRNA	Luciferase	Naphthyl	20.8	6932.9/6932.2	64.1	83.13
Pyr-ssRNA	Luciferase	Pyrenyl	22.6	7006.0/7006.2	42.4	84.80
ssLNA1	Luciferase	5′-LNA	12.8	6604.2/6605.0	–	84.40
ssLNA2	Luciferase	5′- and 3′-LNA	12.9	6575.9/6580.9	–	85.71

[a] A linear gradient condition of CH_3CN shifting the concentrations from 7% to 70% during 40 min in 20 mM TEAA (pH 7.0) using an ODS column.
[b] A saturated solution of 2,4,6-trihydroxyacetophenone in 50 mg/ml diammonium hydrogen citrate in 50% acetonitrile was used as a matrix.
[c] Overall yields of the products were determined by measuring absorbance at 260 nm after HPLC purification.
[d] The ssRNAs conjugated with aromatic compounds were annealed with complementary antisense ssRNAs in a buffer (100 mM NaCl, 20 mM Tris–HCl, pH 7.5), and the melting temperature was detected in absorbance at 260 nm.

luciferase gene expression was dose-dependently suppressed in the siRNA, including Ar-siRNAs and siLNAs, with high potency. Interestingly, the Ar-siRNAs showed stronger inhibitory effects than the unmodified siRNA at concentrations below 2 nM (Fig. 2). In particular, the Ph-siRNA and HyPh-siRNA exhibited strong gene-silencing potency compared with Nap-siRNA and Pyr-siRNA at low concentrations. The siLNA1 exhibited the same level of gene-silencing potency compared with Nap-siRNA and Pyr-siRNA, whereas siLAN2 did not show significantly strong gene silencing; its gene-silencing ability was weaker than those of all Ar-siRNAs.

3.4. Intracellular delivery of Ar-siRNA to HeLa cells

The membrane permeability of siRNAs, including Ar-siRNAs, was investigated in HeLa cells using a confocal fluorescent microscope (Fig. 3). Although the cells treated with unmodified siRNA labeled with FAM at the 5′-end of the antisense strand exhibited fluorescence, the cells treated with Ar-siRNAs labeled with FAM at the 5′-end of the antisense strand exhibited an extremely high fluorescence intensity. In particular, the Ph-siRNA and HyPh-siRNA were localized in the cytoplasm with high abundance. We analyzed the area of each fluorescent image (red or green) using Scion image software (Table 2), and obtained the transfection efficacy of each sample (siRNA and Ar-siRNAs) by dividing the area of fluorescence arisen from Ar-siRNA labeled with FAM (green) by the area of fluorescence arisen from Mitotracker (red). This analysis showed that the transfection efficacy of the Ar-siRNAs to the HeLa cells was 3–5 times higher than that of the unmodified siRNA. Especially, the Ph-siRNA and HyPh-siRNA occupied the wide area of cytoplasm.

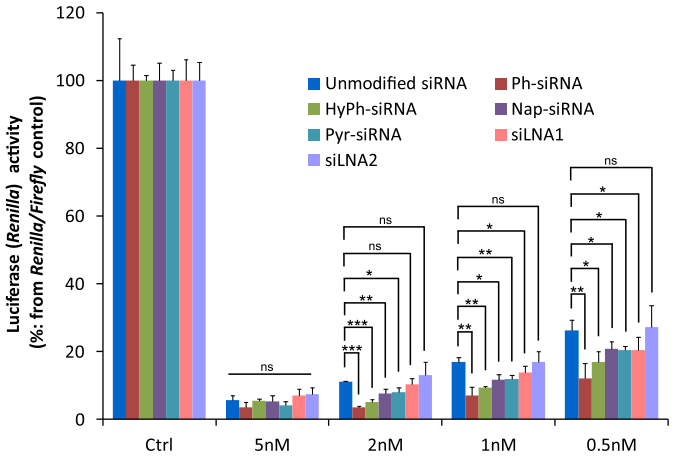

Fig. 2. Comparative gene-silencing efficacy of unmodified siRNA, Ar-siRNAs, and siLNAs targeted to *Renilla* luciferase in HeLa cells in the presence of LF2000. The controls were given only PBS (−). The luminescence of *Renilla* luciferase activity was normalized by the luminescence of *Firefly* luciferase activity. The mean and SD values are from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns = not significant (*t*-test).

4. Discussion

Our study demonstrated that a simply synthesized siRNA with covalently conjugated aromatic compounds would be useful to enhance the potency of RNAi. As a first step, we synthesized several types of Ar-siRNAs by direct conjugation with phenyl, hydroxyphenyl, naphthyl, and pyrenyl derivatives activated by *N*-hydroxysuccinimide ester against *Renilla* luciferase genes (Ph-siRNA, HyPh-siRNA, Nap-siRNA, and Pyr-siRNA). The conjugate position of the aromatic compounds was restricted at the 5′-end of the sense strand, as in our previous reports [24–27]. In HeLa cells, the Ar-siRNAs exhibited a potent RNAi efficacy compared with that of unmodified siRNA. Among these siRNAs, Ph-siRNA and HyPh-siRNA exhibited the strongest gene-silencing efficacy. We assume that aromatic compounds might establish a stacking interaction with the nucleotide base, because the Ar-siRNAs had slightly higher T_m values compared with those of unmodified siRNAs (Table 1). It is surmised that the 5′-end of the conjugated site (the 5′-sense end) of the siRNA would be thermodynamically stabilized compared to the other site (the 3′-sense end). Therefore, it is plausible that the RISC accelerates the selection of the antisense strand of the siRNA, and thus that the Ar-siRNAs increase RNAi potency. Fig. 4 shows our hypothetical model of RISC recognition to Ar-siRNAs. The Ar-siRNAs (5′-sense conjugations) impede the RISC's access to the 5′-sense end (Route A). In that case, the RISC interacts predominantly with the 5′-antisense strand (Route B) and the number of RISC-antisense complexes exceeds that of the RISC-sense complexes. We assumed that siLNA1's high RNAi potency was also attributable to the thermodynamic stability of the 5′-sense site having one LNA molecule. Studies by Ui-Tei et al. [28] and Sano et al. [29] demonstrated that the thermodynamic stability of siRNA termini is a factor in the enhancement of gene-silencing efficacy to accelerate the selection of an antisense strand of siRNA by RISC. SiRNAs consisting of an AU-rich structure at the 3′-end of the sense site and a GC-rich structure at the other site were highly effective for enhancing RNAi. The strand-asymmetry siRNAs also exhibited enhanced RNAi activity with a reduced off-target effect [30,31]. One of the other interesting points is that there is a linear correlation between the RNAi potency and the T_m value of each siRNA. Ph-siRNA and HyPh-siRNA, the modified siRNAs that had slightly increased T_m values compared with unmodified siRNA, exhibited stronger RNAi efficacy than the mildly increased T_m values of Nap-siRNA, Pry-siRNA, and siLNA1. siLNA2, a modified siRNA with the highest thermodynamic stability, did not show significantly strong RNAi efficacy. This suggested that the thermodynamic

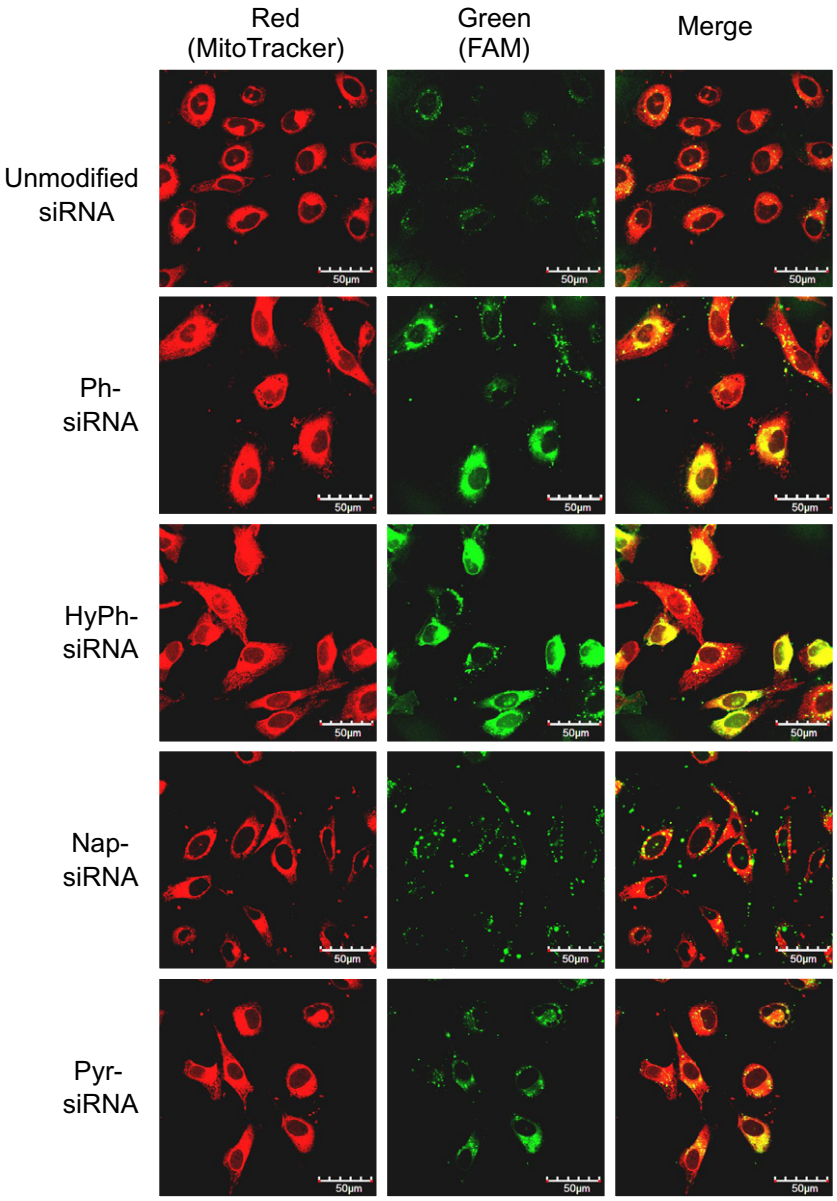

Fig. 3. Confocal microscopic images of HeLa cells incubated for 4 h with unmodified siRNA and Ar-siRNAs in the presence of LF2000. FAM was labeled to the siRNAs at the 5′-end of the antisense strand. The cytoplasm of HeLa cells was visualized by staining with Mito Tracker. Red, fluorescent image from Mito Tracker; Green, fluorescent image from FAM-labeled siRNAs including Ar-siRNAs; Merge, merged image of red and green.

Table 2
Transfection efficacy of siRNA and Ar-siRNAs.

Cells treated with	Area of fluorescence (μm^2)		Transfection efficacy (%)
	Red	Green	
Unmodified siRNA	5757.63	622.62	10.81
Ph-siRNA	5538.22	3137.24	56.65
HyPh-siRNA	6716.99	3847.97	57.29
Nap-siRNA	3586.56	1114.21	31.07
Pyr-siRNA	4107.15	1242.72	30.26

stability of siRNAs influences RNAi potency, which could conceivably affect the RISC recognition and incorporation of the antisense strands of the siRNAs.

High cell membrane permeability and intracellular accumulation of Ar-siRNAs to HeLa cells were also important factors in the enhancement of RNAi efficacy. Although the efficient membrane permeability of siRNA was observed in the presence of LF2000, cytoplasm showed a good deal of bright fluorescence in HeLa cells treated with FAM-labeled Ar-siRNAs. Among these siRNAs, Ph-siRNA and HyPh-siRNA permeated the HeLa cell membrane with high efficiency and were localized in cytoplasm. We assumed these results indicated that the efficient membrane permeability of Ar-siRNAs might be attributable to the satisfactory interaction between LF2000 and aromatic compounds, and that the high yield of accumulation in the cytoplasm of Ar-siRNA, especially for Ph-siRNA and HyPh-siRNA, might be attributable to the positive interaction with the RISC. These fluorescence observations suggested a strong correlation with the RNAi activity of Ar-siRNAs, because Ph-siRNA and HyPh-siRNA exhibited strong RNAi efficacy in our study.

In conclusion, the results suggest in general that the Ar-siRNAs synthesized by our method, especially Ph-siRNA and HyPh-siRNA, are promising for RNAi by virtue of their high stability under physiological conditions, efficient membrane permeability and localization in cytoplasm, and excellent RNAi potency. The Ar-siRNAs

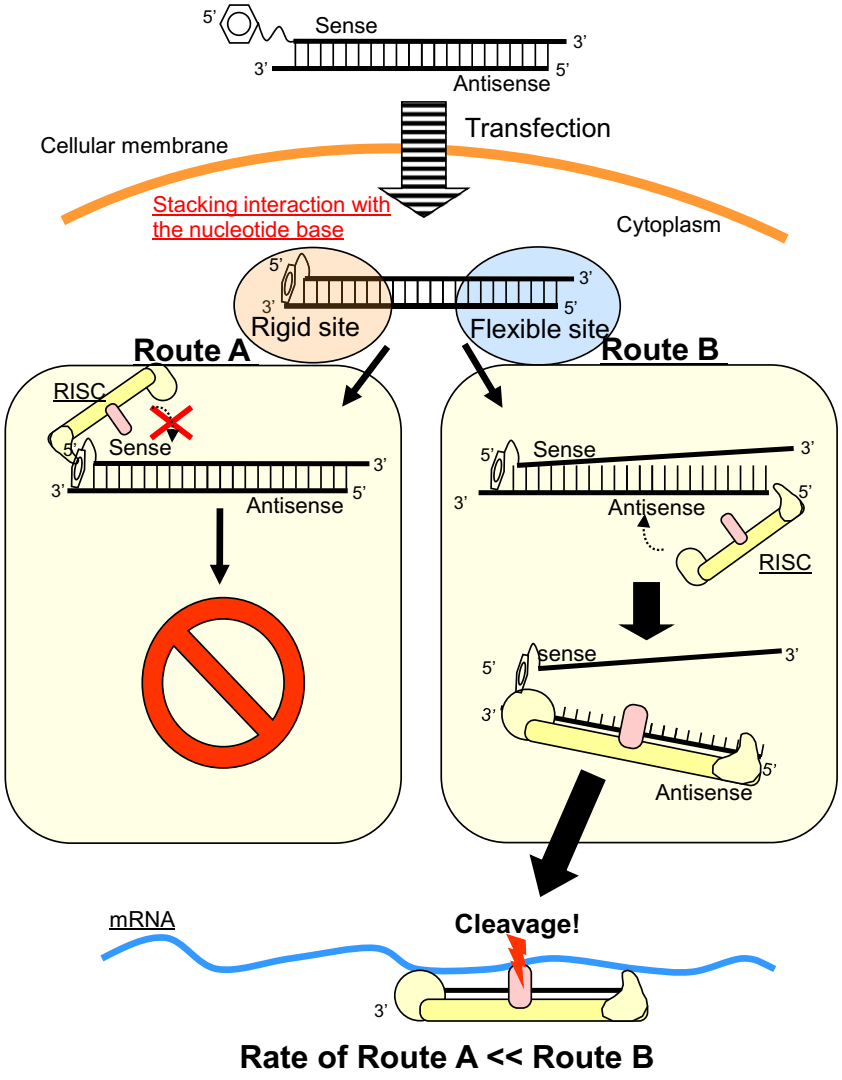

Fig. 4. Hypothetical model of RISC interaction with Ar-siRNAs. The presence of 5′-sense conjugates in Ar-siRNAs impede the access of RISC to the 5′-sense end. Therefore, RISC would interact predominantly with the 5′-antisense strand of Ar-siRNAs and increase the potency of RNAi.

developed in our study can overcome some of the limitations of RNAi technology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.128.

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