Bioconjugate Chemistry

Article

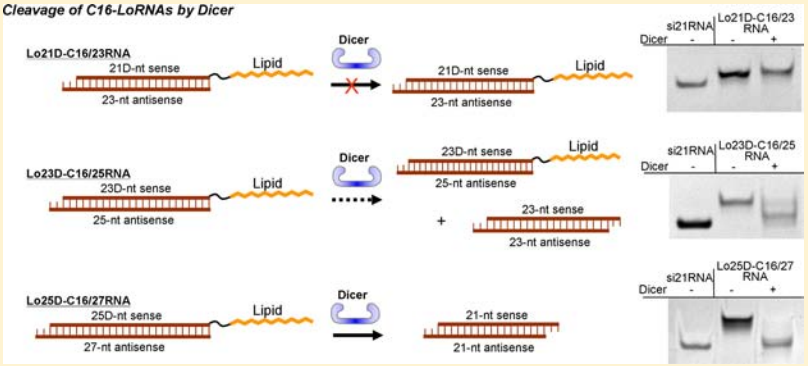
pubs.acs.org/bc

Gene-Silencing Potency of Symmetric and Asymmetric Lipid-Conjugated siRNAs and Its Correlation with Dicer Recognition

Takanori Kubo,*[,†] Kazuyoshi Yanagihara,[‡] Yuichiro Sato,[†] Yoshio Nishimura,[†] Shinichi Kondo,[†] and Toshio Seyama[†]

[†]Faculty of Pharmacy, Yasuda Women's University, 6-13-1 Yasuhigashi, Asaminami-ku, Hiroshima 731-0153, Japan

[‡]Division of Translational Research, National Cancer Center Research Institute, 6-5-1 Kashiwanoha, Kashiwa, Chiba, Japan



ABSTRACT: Three types of siRNAs and three types of left-overhang siRNAs (LoRNAs) were synthesized along with their conjugations with palmitic acid (C16) to investigate the correlation between Dicer recognition and gene-silencing potency. The siRNA types were composed of 21-nucleotide (nt), 23-nt, and 25-nt lengths of sense and antisense strands with a 2-nt overhang at each 3′-end. The three LoRNA types were composed of a 21-nt, a 23-nt, and a 25-nt length of sense strand with a 2-nt DNA at the 3′-blunt-end and a 23-nt, a 25-nt, and a 27-nt length of antisense strand with a 2-nt overhang at the 3′-end. Additionally, each of these siRNAs and LoRNAs was modified with a C16 at the 5′- or 3′-end of the sense strand; these were named C16-siRNAs and C16-LoRNAs, respectively. The siRNAs and C16-siRNAs were barely cleaved by Dicer, and their gene-silencing efficacies were not excellent, contrary to our expectations. In contrast, most of the LoRNAs and C16-LoRNAs became substrates of Dicer, and they showed both strong gene-silencing efficacies and high nuclease resistance. Among the LoRNAs, the 25D-C16/27-nt LoRNA, which is composed of a 25-nt sense strand with a 2-nt DNA conjugated with C16 at the 3′-end and a 27-nt antisense strand with a 2-nt overhang at the 3′-end, showed an excellent gene-silencing effect with high cell membrane permeability and strong resistance against nuclease degradation. Additionally, the Lo25D-C16/27RNA excelled in all three aspects, nuclease resistance, cell membrane permeability, and RNAi efficacy, compared with the cholesterol conjugation. We are certain that Lo25D-C16/27RNA can be useful as a new generation of RNAi molecules with which to overcome some of the limitations of RNAi technology.

INTRODUCTION

RNA interference (RNAi) technology has attracted much attention because of its application to medicines that can potently silence target genes in a sequence-dependent manner.[1−5] RNAi was discovered by Fire et al. using long double-stranded RNA (dsRNA).[1] dsRNAs were cleaved by Dicer, a member of the RNase III family of ribonucleases, into small dsRNAs, called small interfering RNAs (siRNAs) consisting of 21- to 23-nucleotides (nt) with a 2-nt overhang at the 3′-end. These siRNAs triggered potent target gene silencing to induce protein complexes called RNA-induced silencing complexes (RISCs).[6−10] RISCs cleave the target mRNA at a sequence-specific position, guided by the antisense strand of the siRNAs. Thus far, synthetic 21-nt siRNAs have been commonly used for RNAi technology, but they still have the problems of poor membrane permeability and poor nuclease resistance, which limit their therapeutic applicability.[11−17]

Recently, Dicer-substrate siRNAs (DsiRNAs) have gained interest for their greater ability to silence genes compared to normally designed 21-nt siRNAs. Blunt-ended 27-nt DsiRNAs, which are cleaved by a Dicer enzyme leading to the release of 21-nt siRNAs, have been found to exhibit much stronger gene-silencing effects than 21-nt siRNAs.[18−22] Dicer cleaved asymmetrically designed DsiRNAs, such as 25D/27-nt DsiRNA, which are composed of a 25-nt sense strand with a 2-nt DNA blunt-end at the 3′-end and a 27-nt antisense strand with a 2-nt overhang at the 3′-end, leading to the release of a

Received: August 24, 2013
Revised: November 4, 2013
Published: November 26, 2013

   dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

Figure 1. Sequences and structures of siRNAs and LoRNAs. Three types of siRNAs and three types of LoRNAs were designed. The siRNAs were composed of 21-nt, 23-nt, and 25-nt lengths of sense and antisense strands with a 2-nt overhang at each 3′-end, and the LoRNAs were composed of a 21-nt, a 23-nt, and a 25-nt length of the sense strand with a 2-nt DNA blunt-end at the 3′-end and a 23-nt, a 25-nt, and a 27-nt length of antisense strand with a 2-nt overhang at the 3′-end. Ribonucleotides are upper-case, and deoxyribonucleotides are in bold font and lower-case. The letter, p represents 5′-phosphate.

single primary 21-nt siRNA product; moreover, these asymmetrically designed DsiRNAs showed enhanced gene-silencing efficacy and nuclease resistance compared with a normally designed 21-nt siRNA.[23−25] The Dicer-uncleavable asymmetric terminal structures of siRNA, such as 19/21-nt siRNA, which are composed of a 19-nt sense strand and a 21-nt antisense strand with unilateral 2-nt 3′-overhangs on the antisense strand, exhibited enhanced RNAi activity with reduced off-target effects, as did strand asymmetric siRNAs and short hairpin RNAs (shRNAs) having a mismatch near the 5′-end of the antisense strand.[26−31] Although these structurally accurate designed siRNAs, including DsiRNAs, exhibited enhanced RNAi potency, they still have the problems of poor membrane permeability and low resistance against nuclease degradation.

To overcome these problems, a variety of siRNAs conjugated with bioactive molecules have been developed.[32−41] We recently reported that the siRNAs conjugated with aromatic compounds at the 5′-sense end have potent RNAi efficacy with enhanced membrane permeability and antisense selectivity by the RISCs.[32] We also synthesized 27-nt DsiRNA conjugated with a nuclear export signal (NES) peptide, which used the human immunodeficiency virus type-1 (HIV-1) Rev sequence, at the 5′-end of the sense strand, and it enhanced target gene silencing with Dicer-cleaving and cytoplasm accumulation.[33] The covalent conjugation of fatty acids to 21-nt siRNA at the 5′-end, 27-nt DsiRNAs at the 5′-end, or 25D/27-nt DsiRNA at the 3′-end of the sense strand prepared by our simple synthesis strategy showed high membrane permeability and strong RNAi potency.[34−36] Other reports also showed that the covalent conjugation of cholesterol, bile acids, or long-chain fatty acids to siRNAs at the 3′-end of the sense strand mediated siRNA uptake in cells and was applicable *in vivo*.[37−41]

In the present study, we synthesized three types of siRNAs, composed of 21-nt, 23-nt, and 25-nt lengths of sense and antisense strands with a 2-nt overhang at each 3′-end and three types of left overhang siRNAs (LoRNAs), composed of a 21-nt, a 23-nt, and a 25-nt sense strand with a 2-nt DNA blunt-end at the 3′-end and a 23-nt, a 25-nt, and a 27-nt antisense strand with a 2-nt overhang at the 3′-end, as well as their palmitic acid (C16) conjugates (C16-siRNAs and C16-LoRNAs), to investigate the correlation between Dicer recognition and gene-silencing potency. The correlation between nuclease resistance and the gene-silencing potency of LoRNAs including C16-conjugates was also verified. In addition, we performed a comparative RNAi study of C16-conjugated and cholesterol-conjugated LoRNAs. The C16-LoRNAs exhibited the best potential for development into a new generation of RNAi molecules.

■ EXPERIMENTAL PROCEDURES

Design and Synthesis of siRNAs, LoRNAs, and Their C16-Conjugates. All siRNAs and LoRNAs that were designed to target the *Renilla* luciferase gene (Figure 1) were purchased from Integrated DNA Technologies Inc. (IDT, Coralville, IA). Also purchased from IDT were the amino-modified single-strand RNA (ssRNA), including 21, 23, and 25-nt ssRNAs (sense strand) modified with amine at either the 5′-end or the 3′-end for siRNAs, and 19, 21, and 23-nt ssRNA plus 2-nt DNA at the 3′-end (sense strand) modified with amine at only the 3′-end for LoRNAs. To synthesize C16-conjugated ssRNAs, each amino-modified ssRNA (4 nmol in 20 μL water) was reacted

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

Table 1. Characterizations of Single-Strand RNAs (ssRNAs) Conjugated with Palmitic Acid

name	RNA structure	conjugation site	HPLC retention time (min)[a]	MALDI-TOF MS[b] found/calcd
21-nt C16-ssRNA	21-nt RNA	5′-end	29.6	6984.6/6986.4
21-nt ssRNA-C16	21-nt RNA	3′-end	30.2	6987.5/6986.4
23-nt C16-ssRNA	23-nt RNA	5′-end	29.6	7636.4/7636.5
23-nt ssRNA-C16	23-nt RNA	3′-end	29.7	7636.9/7636.5
25-nt C16-ssRNA	25-nt RNA	5′-end	29.3	8270.3/8270.9
25-nt ssRNA-C16	25-nt RNA	3′-end	29.7	8269.9/8270.9
21D-nt ssRNA-C16	19-nt RNA + 2-nt DNA with 5′-phosphate	3′-end	30.4	7034.6/7034.0
23D-nt ssRNA-C16	21-nt RNA + 2-nt DNA with 5′-phosphate	3′-end	29.6	7685.5/7685.0
25D-nt ssRNA-C16	23-nt RNA + 2-nt DNA with 5′-phosphate	3′-end	29.0	8320.3/8319.2

[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.

with 40 nmol palmitic acid *N*-hydroxysuccinimide ester (Sigma-Aldrich, St. Louis, MO) dissolved in 10 μL of *N,N*-dimethylformamide (DMF; Sigma-Aldrich) containing 0.7 μL *N,N*-diisopropylethylamine (DIEA; Sigma-Aldrich) in a 100 μL isopropanol/water (1:1) mixture, for 12 h at room temperature. The ssRNA covalently bound with C16 at the 5′-end (C16-ssRNAs) or 3′-end (ssRNA-C16) was purified by reversed-phase HPLC (RP-HPLC) using an ODS column (4.6 mm × 150 mm, 5 μm) under a linear gradient from 7% to 70% acetonitrile for 40 min in 20 mM triethylammonium acetate (TEAA) (pH 7.0). The molecular weights of all C16-conjugated ssRNAs were confirmed by MALDI-TOF mass spectrometry (Ultraflex; 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. All MALDI-TOF mass spectrometry measurements were carried out in linear-negative mode. The concentrations of all C16-conjugated ssRNAs were calculated using their absorbance at 260 nm as detected spectrophotometrically (V-670 spectrophotometer; Jasco, Tokyo, Japan). The C16-conjugated ssRNAs are characterized in Table 1. The sense strands of C16-conjugated ssRNAs were annealed with antisense strands of ssRNAs in buffer [50 mM Tris-HCl (pH 7.5) and 100 mM NaCl]. The formation of C16-siRNAs or C16-LoRNAs was confirmed by 20% polyacrylamide gel electrophoresis (PAGE; 30 mA, 70 min) and visualized by silver staining (DNA Silver Stain Kit; GE Healthcare, Piscataway, NJ).

***In Vitro* Cleavage of siRNAs, LoRNAs, or Their C16-Conjugates by Recombinant Dicer.** Each type of siRNA or LoRNA, including C16-conjugates (20 pmol), were mixed with 1 U of recombinant Dicer (Gene Therapy Systems Inc., San Diego, CA) in 10 μL of 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 2.5 mM $MgCl_2$. The mixtures were incubated at 37 °C for 12 h, and the reaction was stopped by adding 2 μL of stop solution (Gene Therapy Systems). The reaction products were detected and analyzed by PAGE (20% gel), silver staining, and the LAS4000 imaging system (Fujifilm, Tokyo, Japan).

Cell Cultures and Transfections. HeLa cells, obtained from the RIKEN Bioresource Center Cell Bank (Tsukuba, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% heat-inactivated FBS (Life Technologies, La Jolla, CA), 100 U/mL penicillin (Wako), and 100 μg/mL streptomycin (Wako). To evaluate the RNAi potency of siRNAs or LoRNAs, including the C16-conjugates, targeting the *Renilla* luciferase gene in either the presence or absence of Lipofectamine 2000 (LF2000; Life Technologies), 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. *Renilla* luciferase activity was used to estimate RNAi efficacy. The second reporter, *Firefly* luciferase, was used as a control.

HeLa cells were seeded at 5×10^4 cells/mL in 100 μL of medium in each well of a 96-well multiplate and cultured in a 100% humidified atmosphere (5% CO_2, 37 °C). Twelve hours later, 0.02 μg of the psiCHECK-2 vector was first incubated with 0.2 μL of LF2000 in 10 μL of Opti-MEM (Life Technologies) for 30 min according to the manufacturer's protocol, and then 10 μL of the mixture was added to each well of a 96-well multiplate containing the cells in 90 μL of fresh culture medium without antibiotics. To investigate RNAi in the presence of LF2000, the siRNAs or LoRNAs at different concentrations (2, 1, 0.5, and 0.2 nM) were preincubated with LF2000 as described for the psiCHECK-2 vector. Four hours after the vectors transfected, 10 μL of each preincubated mixture of siRNAs or LoRNAs with LF2000 was added to a well containing 90 μL of fresh culture medium. After another 8 h of incubation, the culture medium was replaced with 100 μL of fresh medium, and the cells were cultured for 48 h to assess RNAi. To investigate the RNAi effect in the absence of LF2000, first the psiCHECK-2 vector was transfected using LF2000 by the same procedure as that described above, and then, the cells were washed three times with culture medium after 4 h of vector transfection. The LoRNAs conjugated with C16 or cholesterol at different concentrations (400, 200, 100, 50, and 25 nM) without any transfection reagents were added to the cells and incubated for 48 h.

Gene Silencing of *Renilla* Luciferase. The efficacy of RNAi against *Renilla* luciferase was evaluated by the Dual-Glo Luciferase Assay System (Promega), which was designed to analyze both *Renilla* and *Firefly* luciferase activities. 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 100 μL of culture medium including siRNAs or LoRNAs. The plates were 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 luminescence arising from the *Renilla* luciferase catalytic reaction was measured in the same way as that described above for *Firefly*

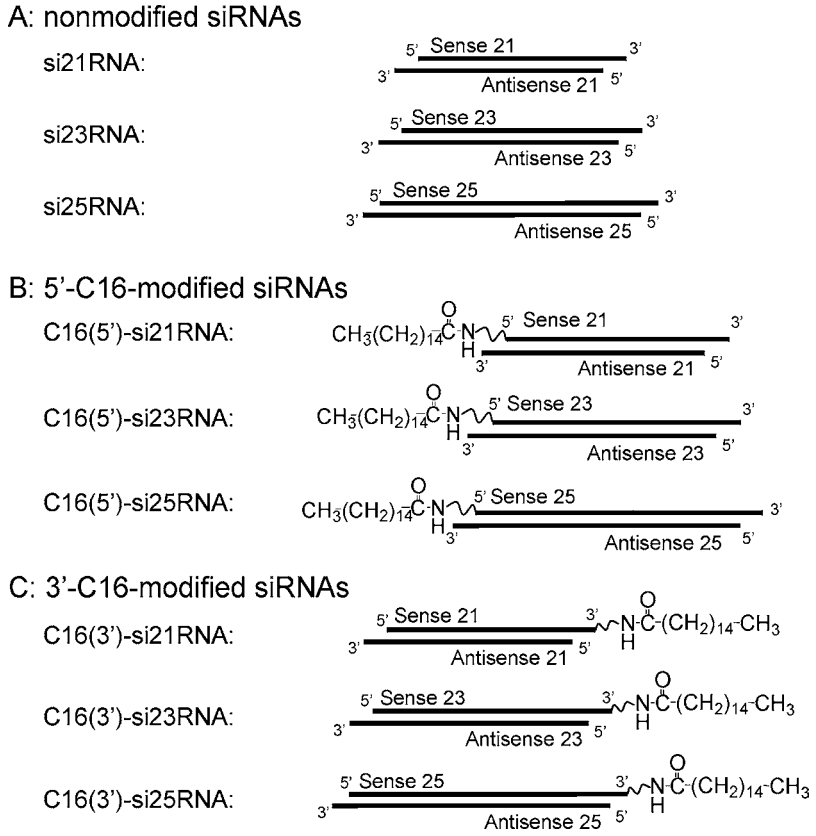


Figure 2. Structures of nonmodified siRNAs (A) and siRNAs conjugated with palmitic acid at the 5′-end (B) or the 3′-end (C). Palmitic acid was covalently conjugated to the si21RNA, si23RNA, and si25RNA at the 5′-end or the 3′-end of the sense strand via amino-linker.

luciferase activity, and the luminescence of *Firefly* luciferase activity was normalized in each well of the 96-well multiplates. The RNAi efficacy of siRNAs or LoRNAs, including C16-conjugates, toward the *Renilla* luciferase was assessed as a percentage of the control (nontreated) sample.

Stability against Nuclease Degradation in Cell-Culture Medium. Ten microliters of each type of LoRNA and C16-LoRNA (200 pmol) was added to 90 μL of DMEM containing 10% heat-inactivated FBS (Invitrogen, La Jolla, CA). The samples were incubated for different intervals (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h) at 37 °C. A 10 μL aliquot was 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 detected and analyzed by 20% PAGE as described in the preceding section.

Intracellular Delivery of LoRNAs, Including C16 or Cholesterol Conjugates. To prepare fluorescence (FAM)-labeled LoRNAs, the FAM-labeled antisense 23-, 25-, and 27-nt ssRNA was annealed with either palmitic acid conjugated or nonmodified sense 19-, 21-, and 23-nt ssRNA plus 2-nt DNA at the 3′-end, respectively, in an annealing buffer as in the protocols described above. The cholesterol-conjugated sense 23-nt ssRNA plus 2-nt DNA at the 3′-end was also annealed with FAM-labeled antisense 27-nt ssRNA.

To deliver the prepared LoRNAs intracellularly, 200 pmol of each LoRNA, including C16 or cholesterol conjugation labeled with FAM, was incubated with 2 μL of LF2000 in 100 μL of Opti-MEM diluted twice for 30 min at room temperature. Then, 100 μL of the mixture was added to 900 μL of culture of HeLa cells (5×10^4 cells) supplemented with 10% heat-inactivated FBS (Life Technologies), 100 U/mL penicillin (Wako), and 100 μg/mL streptomycin (Wako) and incubated for 6 h in the dark under a humidified atmosphere (5% CO_2, 37 °C). The cells were washed several times with fresh medium, and an intracellularly incorporated amount of LoRNAs labeled with FAM in cells was examined under a fluorescent confocal microscope (IX70; Olympus, Tokyo, Japan).

Another approach was used to investigate the intracellular delivery of LoRNAs, including C16 or cholesterol conjugation labeled with FAM, by flow cytometry (FACSAria; BD Biosciences, Franklin Lakes, NJ). The forward- and side-scatter parameters were adjusted to accommodate the inclusion of each dissociated cell line with the aid of FAM as a marker. Five thousand cells were analyzed, and no cells were excluded from the analysis. Data were collected and analyzed using FACSDiva software (BD Bioscience).

RESULTS

Correlation between Dicer Processing and RNAi Efficacy of Each Type of siRNA and C16-siRNA. Three siRNA lengths, 21-, 23-, and 25-nt, each with a 2-nt overhang at the 3′-end, were designed to target the *Renilla* luciferase gene, and C16 was covalently bound to either the 5′-end or the 3′-end of the sense strand of each of these siRNAs (Figure 2). We investigated whether the nonmodified siRNAs and C16-siRNAs were substrates of Dicer leading to the release of the 21-nt siRNAs (Figure 3A). Nonmodified 21-nt siRNA (si21RNA) and its C16-conjugates (C16(5′)-si21RNA and C16(3′)-si21RNA), showed the same mobility on PAGE in either the presence or absence of the Dicer enzyme. Thus, the Dicer enzyme did not recognize the siRNA with the 21-nt nucleotide whether or not it was modified with the C16.

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

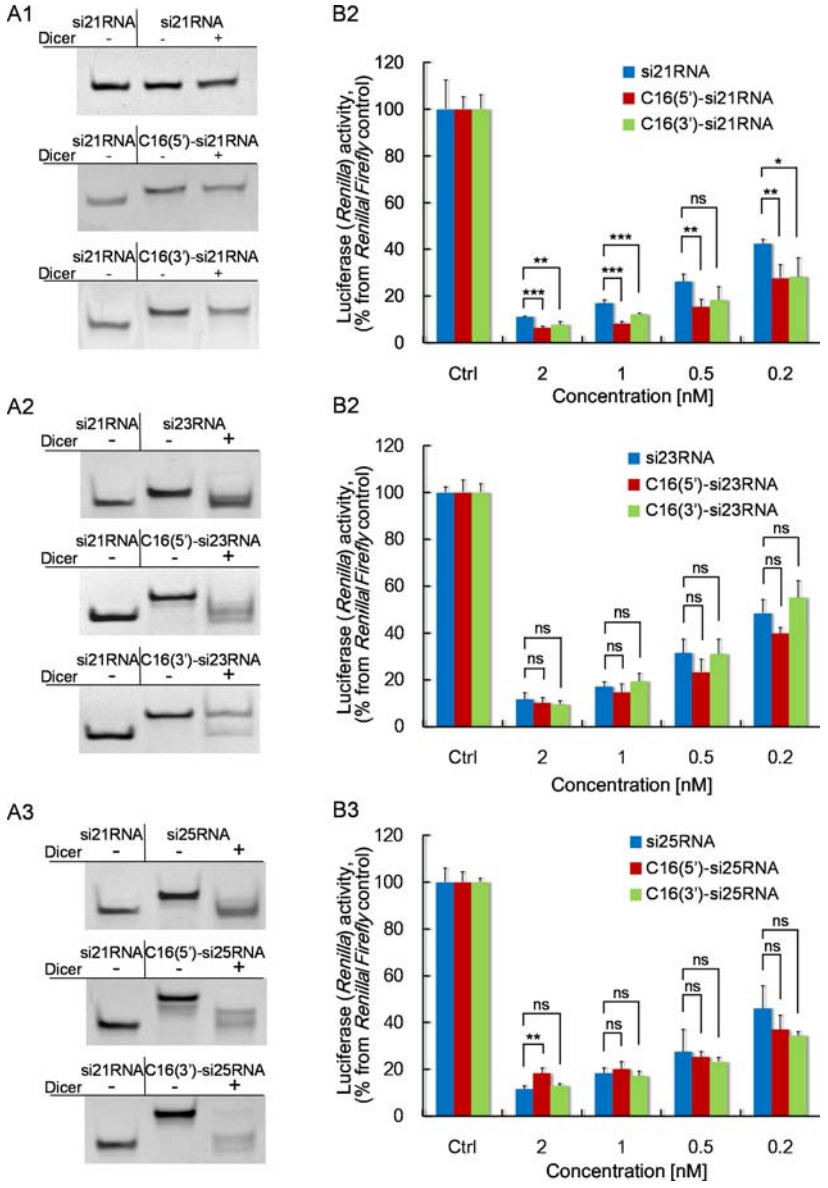


Figure 3. Correlative study between Dicer cleavage and RNAi efficacy using each type of siRNAs and C16-siRNAs. (A) Dicer cleavage of siRNAs and C16-siRNAs. The si21RNA (A1), si23RNA (A2), si25RNA (A3), and their C16-conjugates at either the 5′-end or 3′-end were reacted with the recombinant Dicer enzyme for 12 h at 37 °C. The reaction products were electrophoresed on 20% PAGE and visualized by silver staining. (B) RNAi efficacies of siRNAs and C16-siRNAs. The si21RNA (B1), si23RNA (B2), si25RNA (B3), and their C16-conjugates at either the 5′- or 3′-end (2, 1, 0.5, and 0.2 nM) were transfected by LF2000 to HeLa cells. Controls were given only PBS (−). RNAi efficacies were evaluated to detect the luminescence of *Renilla* luciferase activity, which was normalized by the luminescence of *Firefly* luciferase activity, after 48 h of incubation. The mean and SD are from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant (t test).

Dicer partly cleaved the nonmodified 23-nt siRNA (si23RNA) and its C16-conjugates (C16(5′)-si23RNA and C16(3′)-si23RNA). si23RNA was a substrate of Dicer, leading to the release of most 21-nt siRNA products, but different products also appeared with the 21-nt siRNA. C16(5′)-si23RNA was also a substrate of the Dicer enzyme, but the same rate with different products, including 21-nt siRNA, was found. Although the Dicer enzyme cleaved a part of C16(3′)-si23RNA, Dicer did not recognize most of this siRNA, and thus the same mobility was observed on PAGE either in the presence or absence of the Dicer enzyme.

Nonmodified 25-nt siRNA (si25RNA) was cleaved to the 21-nt siRNA product after reaction with Dicer. Although the si25RNA conjugated with C16 at the 5′-sense end (C16(5′)-si25RNA) was also a Dicer substrate, there were two different products after the reaction: one was the 21-nt siRNA, while the other seemed to be the 23-nt siRNA. The si25RNA conjugated with C16 at the 3′-sense end (C16(3′)-si25RNA) showed several different products after the reaction with Dicer. At least one product appeared to be the 21-nt siRNA, but the others seemed to be a 22−23-nt siRNA. A noncleavable product, the same as that produced from mobility on PAGE in the absence of Dicer, was also found after the reaction.

The RNAi efficacies of each type of siRNA and C16-siRNA were evaluated using a luciferase gene reporter assay in the presence of LF2000 (Figure 3B). Each type of siRNA and C16-siRNA exhibited dose-dependent suppression of the *Renilla* luciferase activity. In a comparison between the si21RNA and

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

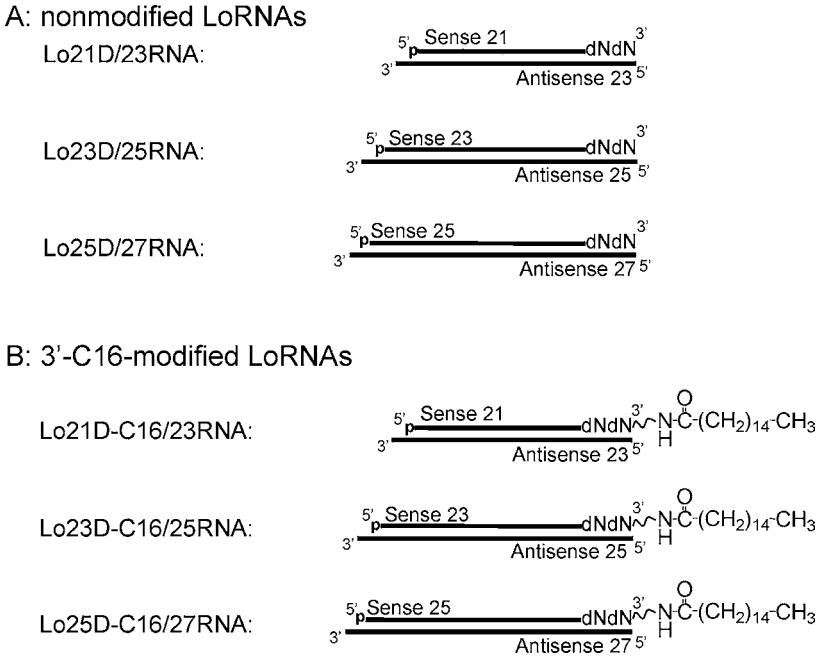


Figure 4. Structures of nonmodified LoRNAs (A) and LoRNAs conjugated with palmitic acid at the 3′-end (B). Palmitic acid was covalently conjugated to the Lo21D/23RNA, Lo23D/25RNA, and Lo25D/27RNA at the 3′-end of the sense strand via an amino-linker.

its C16-conjugates, C16-si21RNAs exhibited higher RNAi efficacy than si21RNA, although all of the *Renilla* luciferase activities were reduced from the control value (nontreated cells) by the RNAi of si21RNA. It was noteworthy that C16-(5′)-si21RNA exhibited a slightly higher inhibitory effect than C16-(3′)-si21RNA. In a comparison between si23RNA and its C16-conjugates, C16-(5′)-si23RNA showed the strongest RNAi efficacy. The RNAi efficacy of C16(3′)-si23RNA was the same as or lower than that of si23RNA. si25RNA and its C16-conjugates exhibited almost the same level of gene-silencing efficacy. These results suggest that the structure of siRNA after the reaction with Dicer is important for obtaining potent RNAi efficacy, whether or not it becomes a substrate of Dicer.

Correlations between Dicer Processing and the RNAi Efficacy of Each Type of LoRNA and C16-LoRNA. We synthesized three types of LoRNAs: Lo21D/23RNA, composed of a 21-nt sense strand with 2-nt DNA at the 3′-blunt-end and a 23-nt antisense strand with a 2-nt 3′-overhang; Lo23D/25RNA, composed of a 23-nt sense strand with 2-nt DNA at the 3′-blunt-end and a 25-nt antisense strand with a 2-nt 3′-overhang; and Lo25D/27RNA, composed of a 25-nt sense strand with 2-nt DNA at the 3′-blunt-end and a 27-nt antisense strand with a 2-nt 3′-overhang (Figure 4A). These LoRNAs were conjugated with C16 at the 3′-end of the sense strand via an amino-modified linker (Figure 4B). The C16-conjugated Lo21D/23RNA, Lo23D/25RNA, and Lo25D/27RNA were named Lo21D-C16/23RNA, Lo23D-C16/25RNA, and Lo25D-C16/27RNA, respectively.

We next investigated whether the LoRNAs and C16-LoRNAs were substrates of Dicer cleaved to the 21-nt siRNA (Figure 5A). Lo21D/23RNA and Lo21D-C16/23RNA were not substrates of the Dicer enzyme, as they showed the same mobility on PAGE in either the presence or absence of the Dicer enzyme. Most of the Lo23D/25RNA was cleaved, leading to the release of the 21-nt siRNA, but part of it showed the same mobility on PAGE in the absence of the Dicer enzyme. Lo23D-C16/25RNA also became a Dicer substrate, but in this case a product other than 21-nt siRNA was obtained. Lo25D/27RNA and Lo25D-C16/27RNA were almost completely cleaved by Dicer, leading to the release of a 21-nt siRNA.

The RNAi efficacies of each type of LoRNA and C16-LoRNA were evaluated using the same protocols as those used for the siRNAs experiment described above (Figure 5B). *Renilla* luciferase gene expression was dose-dependently suppressed in all samples (LoRNAs and its C16-conjugates) with high potency. Lo21D/23RNA and Lo25D/27RNA, as well as their C16-conjugates, suppressed *Renilla* luciferase gene expression with high potency in HeLa cells. In particular, Lo21D-C16/23RNA and Lo25D-C16/27RNA exhibited excellent target gene silencing, and their RNAi efficacies were superior to those of nonmodified LoRNAs. Specifically, Lo21D-C16/23RNA and Lo25D-C16/27RNA exhibited 90.1% and 88.5% knockdown of the *Renilla* luciferase gene, respectively, whereas Lo21D/23RNA and Lo25D/27RNA exhibited 84.2% and 83.7% knockdown, respectively, at a concentration of 0.5 nM. Although *Renilla* luciferase gene expression in HeLa cells was dose-dependently suppressed after treatment with different concentrations of Lo23D/25RNA or Lo23D-C16/25RNA, the gene-silencing efficacies were lower than those in cells treated with Lo21D/23RNA, Lo25D/27RNA, or the C16-conjugate of either. Lo23D-C16/25RNA also had a stronger inhibitory effect than Lo23D/25RNA at all concentrations used in this study. Thus, the inhibitory effects of the LoRNAs conjugated with C16 at the 3′-end of the sense strand were superior to those of the nonmodified LoRNAs. Additionally, higher RNAi potency was obtained in the C16-conjugated LoRNAs than in the C16-conjugated siRNAs. Nevertheless, the gene-silencing efficacies of the LoRNAs and C16-LoRNAs also influenced their structure after the reaction with Dicer, whether or not they became a substrate of Dicer.

Nuclease Resistance of Each Type of LoRNA and C16-LoRNA. We investigated the nuclease resistance of each type of LoRNA and C16-LoRNA in cell culture medium containing

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

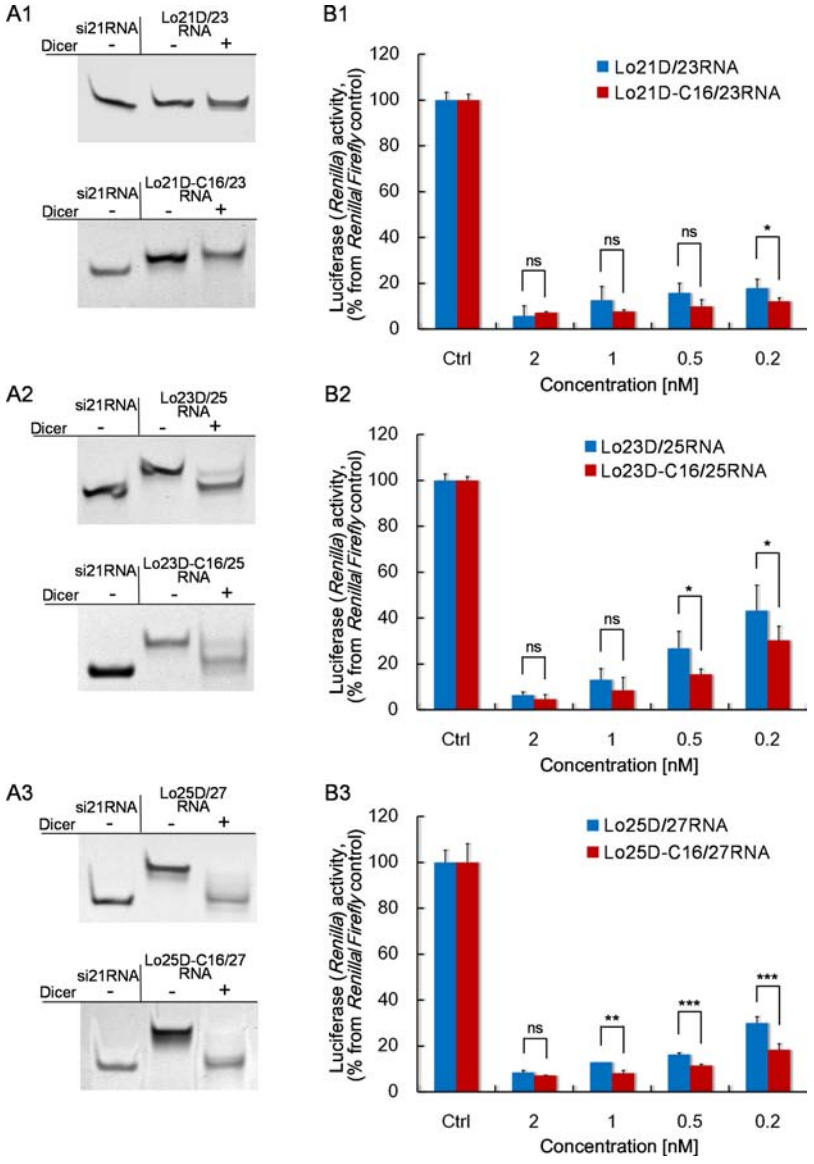


Figure 5. Correlative study between Dicer cleavage and RNAi efficacy using each type of LoRNA and C16-LoRNA. (A) Dicer cleavage of LoRNAs and C16-LoRNAs. The Lo21D/23RNA (A1), Lo23D/25RNA (A2), Lo25D/27RNA (A3), and their C16-conjugates at the 3′-end were reacted with the recombinant Dicer enzyme for 12 h at 37 °C. The reaction products were electrophoresed on 20% PAGE and visualized by silver staining. (B) RNAi efficacies of LoRNAs and C16-LoRNAs. The Lo21D/23RNA (B1), Lo23D/25RNA (B2), Lo25D/27RNA (B3), and their C16-conjugates at the 3′-end (2, 1, 0.5, and 0.2 nM) were transfected by LF2000 to HeLa cells. Controls were given only PBS (−). RNAi efficacies were evaluated to detect the luminescence of *Renilla* luciferase activity, which was normalized by the luminescence of *Firefly* luciferase activity, after 48 h of incubation. The mean and SD are from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant (t test).

10% FBS at different incubation times (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h) (Figure 6A). The stability of the si21RNA in culture medium was also investigated as a standard control using the same protocol. The si21RNA was degraded immediately and completely disappeared after about 5 h of incubation. In contrast, all LoRNAs and C16-LoRNAs showed greater stability against nuclease than si21RNA. A comparison among LoRNAs showed that the survival of intact LoRNAs depended on molecule length. Thus, Lo25D/27RNA showed the greatest stability, whereas Lo21D/23RNA was degraded the earliest. The stability of C16-LoRNAs in culture medium had the same tendency as that of LoRNAs; thus, Lo25D-C16/27RNA had the highest yield of intact molecules, whereas the degradative rate of Lo21D-C16/23RNA was the fastest in the C16-LoRNAs. It should be noted that all C16-LoRNAs showed excellent stability compared with the nonmodified LoRNAs and that Lo25D-C16/27RNA showed the strongest resistance among all of the samples.

To investigate the RNAi efficacy of surviving LoRNAs or C16-LoRNAs after nuclease treatment, each aliquot sample described above was examined in a gene-silencing study targeted to the *Renilla* luciferase gene (Figure 6B). The aliquot samples at different incubation times (0, 1, 4, 8, 12, 24, and 48 h) were prepared to 1 nM concentrations of LoRNAs or C16-LoRNAs, which were attributed to the concentration before nuclease treatment, and the RNAi efficacy of each was investigated in the presence of LF2000 in the same way as described above for the *Renilla* luciferase assay system. Lo21D/23RNA and its C16-conjugates exhibited dramatic attenuation of RNAi efficacy. In contrast, the aliquot samples of Lo25D/

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

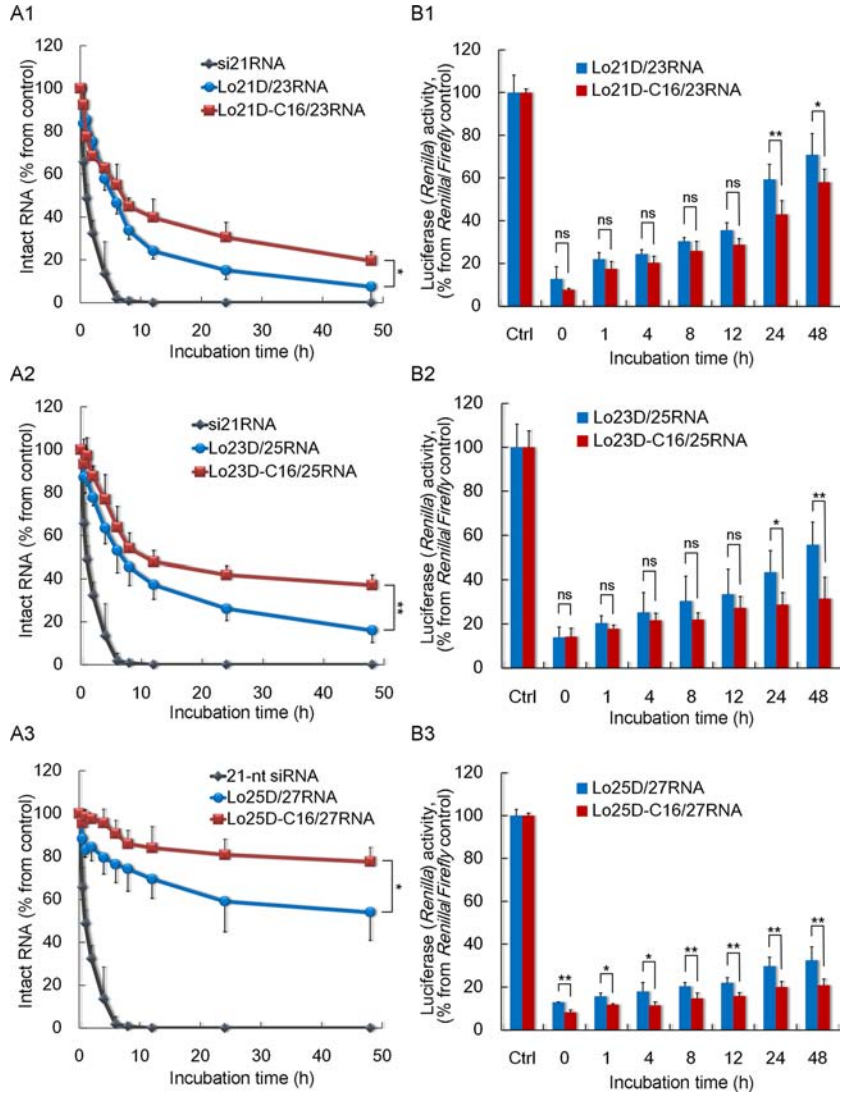


Figure 6. Nuclease resistance and its relationship to the RNAi efficacy of each type of LoRNAs and C16-LoRNAs. (A) Nuclease resistance of LoRNAs and C16-LoRNAs. The Lo21D/23RNA (A1), Lo23D/25RNA (A2), Lo25D/27RNA (A3), and their C16-conjugates at the 3′-end were incubated in culture medium containing 10% FBS at 37 °C for 0, 0.5, 1, 2, 4, 6, 8, 12, 24, or 48 h, and aliquots were analyzed on 20% PAGE. The mean and SD are from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ at 48 h of incubation time (t test). (B) RNAi efficacy of LoRNAs and C16-LoRNAs after treatment with 10% FBS. The aliquots containing LoRNAs and C16-LoRNAs (1 nM) after treatment with 10% FBS for 0, 1, 4, 8, 12, 24, and 48 h were investigated for RNAi efficacy in the presence of LF2000. The mean and SD are from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns = not significant (t test).

27RNA and Lo25D-C16/27RNA maintained an inhibitory effect even though the sample was incubated for a long time. In particular, Lo25D-C16/27RNA maintained strong gene-silencing efficacy throughout a long incubation time and exhibited 80% knockdown of *Renilla* gene expression using an aliquot sample after 48 h of treatment. There was a linear correlation between high nuclease resistance and the longevity of the RNAi efficacy of LoRNAs and C16-LoRNAs.

Intracellular Delivery of Each Type of LoRNA and C16-LoRNA. The cell membrane permeability of LoRNAs and C16-LoRNAs was investigated in HeLa cell lines in the presence of LF2000 using confocal microscopy and flow cytometry. In the observation by confocal microscopy, the cells treated with C16-LoRNAs, labeled with FAM, exhibited brighter fluorescence with greater cytoplasm accumulation than those treated with nonmodified LoRNAs (Figure 7). Thus, the C16 conjugates showed enhanced cell membrane permeability and cytoplasm accumulation in culture cells, as predicted by the properties of palmitic acid. The results of flow cytometric analysis showed the same tendency as the microscope observations. In the flow cytometric analysis, the area of the histogram was separated into four populations (a−d) according to the fluorescence intensity. The histograms of the cells treated with C16-LoRNAs labeled with FAM exhibited higher fluorescence-intensity populations than the histograms of the cells treated with nonmodified LoRNAs. In a comparison among C16-LoRNAs, the cells treated with Lo25D-C16/27RNA showed the brightest fluorescence both by the microscopic and flow cytometric analyses. Lo25D/27RNA also showed the highest membrane permeability among LoRNAs. It seemed that the increased membrane permeability of the C16-LoRNAs compared with the LoRNAs was attributable to the satisfactory interaction of the former with both the cellular membrane and palmitic acid. In addition, our results suggested that the intracellular stabilities

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045−2057

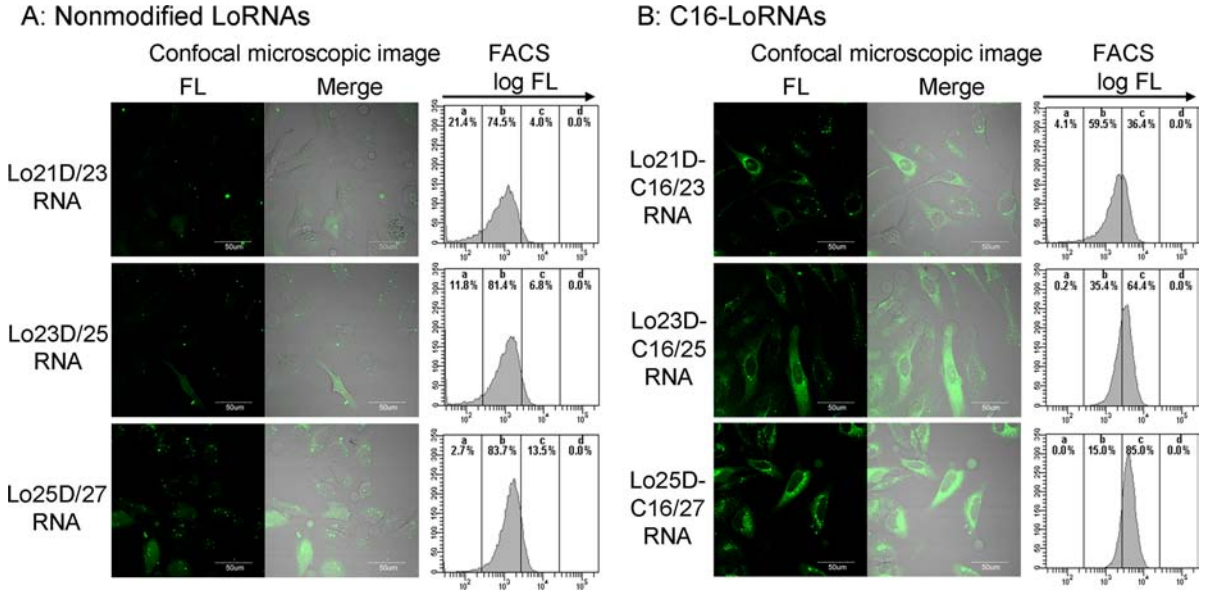


Figure 7. Confocal microscopy images and flow cytometric analysis of HeLa cells incubated for 6 h with nonmodified LoRNAs (A) or C16-LoRNAs (B) (200 nM) labeled with FAM, in the presence of LF2000. FL, fluorescence image; Merge, merged image of FL and transmission images; and FACS, flow cytometric analysis. In the flow cytometric analysis, the logarithm of the fluorescence intensity is shown on the horizontal axis, and the number of cells is shown on the longitudinal axis. Cell population is separated into four parts (a–d) along with the fluorescence intensity.

of the C16-LoRNAs and LoRNAs depended on their molecular length, which appeared to be similar to the above-described results for the nuclease stability in culture medium. Accordingly, C16-25D/27RNA had two essential elements for RNAi potency: a high membrane permeability due to palmitic acid conjugation and a strong stability against nuclease degradation.

Comparative Study of Lo25D-C16/27RNA and Lo25D-Chol/27RNA. We performed a comparative study of Lo25D-C16/27RNA and cholesterol-conjugated Lo25D/27RNA (Lo25D-Chol/27RNA) to investigate their RNAi efficacy, nuclease stability, and membrane permeability (Figure 8). The cholesterol-conjugated ssRNA was purchased from IDT and annealed with antisense ssRNA to obtain Lo25D-Chol/27RNA (Figure 8A). Lo25D-Chol/27RNA was first examined to determine whether it was a substrate of Dicer, and Dicer cleaved it completely, leading to the release of a 21-nt siRNA (Figure 8B). Thus, Lo25D/27RNA could be a substrate of Dicer even though fatty acid or cholesterol was attached at the 3′-end of the sense strand.

We next performed a comparative study of the RNAi potency of the Lo25D-C16/27RNA and Lo25D-Chol/27RNA against the *Renilla* luciferase gene in either the presence or absence of LF2000. In the presence of LF2000, *Renilla* luciferase gene expression was dose-dependently suppressed with high potency in both Lo25D-C16/27RNA and Lo25D-Chol/27RNA (Figure 8C1). Furthermore, Lo25D-C16/27RNA exhibited stronger gene-silencing efficacy than Lo25D-Chol/27RNA.

In the absence of LF2000, Lo25D-Chol/27RNA was verified to have strong RNAi efficacy at a concentration of 400 nM, but it showed lower gene-silencing activity than Lo25D-C16/27RNA at concentrations between 100 nM and 400 nM (Figure 8C2). The nonmodified Lo25D/27RNA did not inhibit *Renilla* luciferase activity at any concentration, even as high as 400 nM (data not shown).

We also compared nuclease resistance between Lo25D-C16/27RNA and Lo25D-Chol/27RNA in a cell culture medium containing 10% FBS at different incubation times (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h) (Figure 8D). Although Lo25D-Chol/27RNA had excellent stability in culture medium, Lo25D-C16/27RNA showed slightly higher resistance than Lo25D-Chol/27RNA.

The membrane permeability of Lo25D-C16/27RNA or Lo25D-Chol/27RNA was investigated in HeLa cells in the presence of LF2000 using confocal microscopy and flow cytometry. In the observation by confocal microscopy, although the cells treated with Lo25D-Chol/27RNA exhibited bright fluorescence with cytoplasm accumulation in the presence of LF2000 (Figure 8E), Lo25D-C16/27RNA labeled with FAM showed higher membrane permeability than Lo25D-Chol/27RNA. Flow cytometric analysis showed the same tendency in the results as the microscope observations. The histograms of the cells treated with Lo25D-C16/27RNA or Lo25D-Chol/27RNA labeled with FAM exhibited populations with high fluorescence intensity. Especially, the cells treated with Lo25D-C16/27RNA labeled with FAM were distributed in higher fluorescence intensity populations than the cells treated with Lo25D-Chol/27RNA in the histograms. Accordingly, the C16 conjugates showed enhanced cell membrane permeability in culture cells, as predicted by the properties of palmitic acid.

■ DISCUSSION

In this study, three different types of siRNAs (si21RNA, si23RNA, and si25RNA) and LoRNAs (Lo21D/23RNA, Lo23D/25RNA, and Lo25D/27RNA), as well as their C16-conjugates, were synthesized to explore their influences on gene-silencing potency and a Dicer substrate.

We initially performed a comparative study of Dicer cleavage and gene silencing using siRNAs and C16-siRNAs. Although the series of si21RNAs (si21RNA, C16(5′)-si21RNA, and C16(3′)-siRNAs) were not cleaved by Dicer, high RNAi potencies were verified. Especially, C16(5′)-si21RNA exhibited

dx.doi.org/10.1021/bc400391n | *Bioconjugate Chem.* 2013, 24, 2045–2057

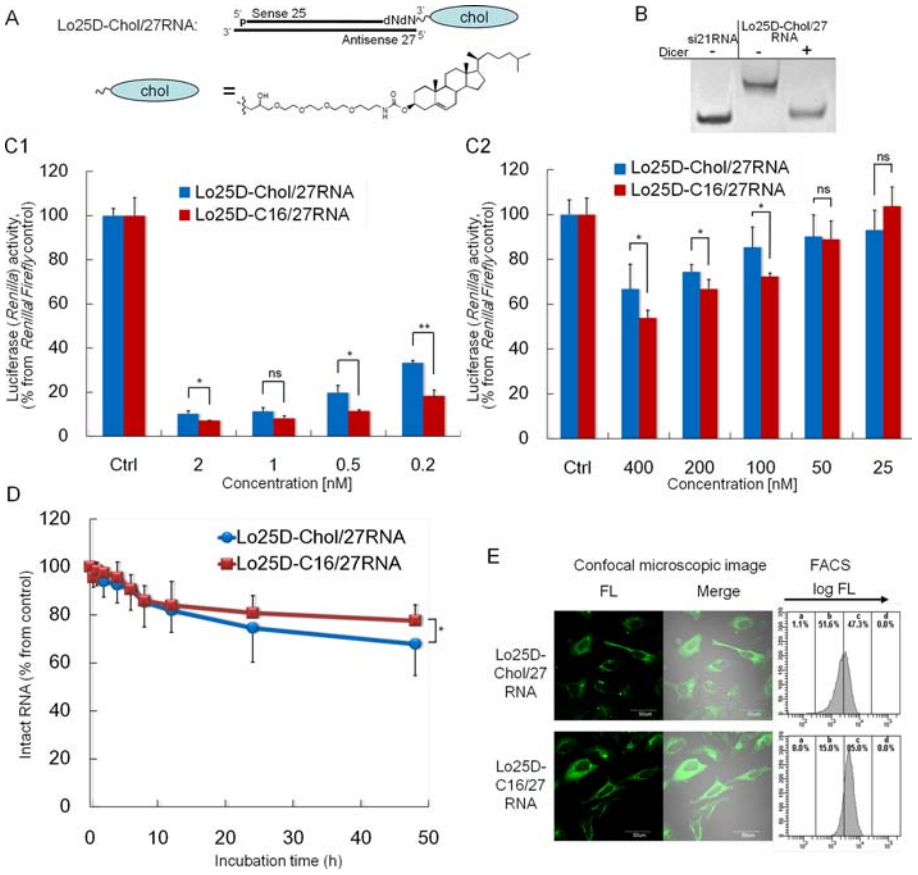


Figure 8. Comparative study of Lo25D-C16/27RNA and Lo25D-Chol/27RNA using Dicer cleavage, RNAi efficacy, nuclease resistance, and cell membrane permeability. (A) Structure of Lo25D-Chol/27RNA. (B) Dicer cleavage study of Lo25D-Chol/27RNA. (C) Comparative RNAi efficacy of Lo25D-C16/27RNA and Lo25D-Chol/27RNA in the presence (C1) or absence (C2) of LF2000. The mean and SD are from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns = not significant (t test). (D) Comparative nuclease resistance of Lo25D-C16/27RNA and Lo25D-Chol/27RNA. Lo25D-C16/27RNA and Lo25D-Chol/27RNA were incubated in culture medium containing 10% FBS at 37 °C for 0, 0.5, 1, 2, 4, 6, 8, 12, 24, or 48 h, and aliquots were analyzed on 20% PAGE and RP-HPLC. (E) Confocal microscopy images and flow cytometric analysis of HeLa cells incubated for 6 h with Lo25D-C16/27RNA or Lo25D-Chol/27RNA (200 nM) labeled with FAM, in the presence of LF2000. FL, fluorescence image; Merge, merged image of FL and transmission images; and FACS, flow cytometric analysis. In the flow cytometric analysis, the logarithm of the fluorescence intensity is shown on the horizontal axis, and the number of cells is shown on the longitudinal axis. Cell population is separated into four parts (a–d) along with the fluorescence intensity.

the strongest inhibition of gene expression among all siRNAs and C16-siRNAs. In the literature, lipid-conjugated siRNA has been reported to have high RNAi potency. The lipids, including cholesterol, bile acids, or long-chain fatty acids, conjugated with siRNAs facilitated cellular penetration of the siRNAs through their interaction with the lipid bilayer of the cell membrane, lipoprotein particles, lipoprotein receptors, or transmembrane proteins.[38] Because the lipoprotein particles were expected to protect siRNA from nuclease degradation, these lipophilic siRNAs were also highly stable against nuclease degradation. The si23RNA, si25RNA, and their C16-conjugates became substrates of Dicer, but the products after the reaction with Dicer included not only single 21-nt siRNA but also siRNAs of various lengths. In addition, Dicer did not recognize some of the si23RNAs and si25RNAs conjugated with C16 restricted at the 3′-end of the sense strand. These results imply that a 2-nt 3′-overhang structure is an essential region for siRNA recognition by the Dicer enzyme. Other reports have also described that the 2-nt 3′-overhang structure is important for the recognition of the PAZ domain, which is an essential domain of Dicer, and consequently, Dicer measures from the 3′-end of the siRNA, leading to the release of 21-nt siRNA.[42−45] The RNAi potency of the si23RNA, si25RNA, and C16-conjugates of each exhibited lower gene-silencing efficacy than si21RNA and its C16-conjugates. It is noteworthy that C16(3′)-si23RNA, which existed in intact molecules after the reaction with Dicer, showed the weakest gene silencing among the siRNAs and C16-siRNAs. The results of gene silencing described above suggested that potent RNAi efficacy correlates highly with their Dicer-cleaving behavior. In addition, the structure of siRNA after the reaction with Dicer is important for obtaining potent RNAi efficacy, whether or not it becomes a substrate of Dicer. Kim et al. also reported that the cleavage of a 27-nt DsiRNA by Dicer inside cells could result in a variety of distinct 21-nt siRNA products and that a mix of these possible 21-nt siRNAs is significantly more potent than a specific 21-nt siRNA.[18] They also discussed that a 27-nt DsiRNA had stronger RNAi efficacy than seven different synthesized 21-nt siRNA sets that could be derived from the cleavage of a 27-nt DsiRNA by Dicer. The 25D/27-nt DsiRNAs were cleaved by Dicer leading to the release of a single primary 21-nt siRNA product and exhibited potent gene-silencing effects.[23−25] Other strand asymmetry siRNAs also exhibited enhanced RNAi activity with reduced off-target effects.[26−31]

dx.doi.org/10.1021/bc400391n | Bioconjugate Chem. 2013, 24, 2045−2057

Thus, the structurally accurate designed siRNAs showed enhanced gene-silencing efficacy and nuclease resistance compared with symmetric 21-nt siRNA. Therefore, we synthesized three different types of DsiRNA motifs, which are asymmetric duplex RNAs composed of a sense strand with a 3′-blunt-end and an antisense strand with a 2-nt 3′-overhang (LoRNAs), and also synthesized their C16-conjugates (C16-LoRNAs), expecting strong RNAi potency, high membrane permeability, and high nuclease stability. The C16 conjugation to LoRNAs was restricted to the 3′-end of the sense strand so that we could examine the importance of the 2-nt overhang recognized by Dicer. Comparative studies of Dicer cleavage, RNAi efficacy, nuclease resistance, and cell membrane permeability using each type of LoRNA and C16-LoRNA were performed. Lo25D-C16/27RNA showed the best performance for RNAi. Because Dicer recognized this molecule, leading to the release of a single 21-nt siRNA product, it had superior gene-silencing efficacy while exhibiting both excellent cell membrane permeability and the strongest resistance against nuclease degradation among all LoRNAs. The other LoRNAs and C16-LoRNAs also showed high potential as RNAi's. Although Dicer did not recognize Lo21D/23RNA and Lo21D-C16/23RNA, both molecules exhibited strong RNAi potency. During their construction, Lo21D/23RNA and Lo21D-C16/23RNA showed weak resistance against nuclease degradation and low intracellular abundance compared with Lo25D/27RNA and Lo25D-C16/27RNA, respectively. These results suggest that, although short-length LoRNAs such as Lo21D/23RNA are suitable for short-term RNAi treatment, they may be unsuitable for long-term RNAi treatment and *in vivo* experiments. 23D/25RNA and its C16-conjugate demonstrated good properties as RNAi's in Dicer cleavage and good RNAi potency, nuclease resistance, and membrane permeability, but they were not superior to Lo25D-C16/27RNA in all ways. In addition, Lo25D-C16/27RNA had stable gene-silencing efficacy with high potency even after long-term treatment with nuclease. Thus, the strong resistance of siRNA against nuclease degradation is an essential element for longevity in RNAi therapy. Therefore, Dicer-cleavable lipid-conjugated siRNAs, such as Lo25D-C16/27RNA in this study, could be more prominent than Dicer-uncleavable lipid-conjugated siRNAs, such as Lo21D-C16/23RNA.

We next investigated the biological properties of Lo25D-Chol/27RNA. It was reported that covalent conjugation of cholesterol to siRNAs mediated siRNA uptake in cells *in vitro* and *in vivo* and that these siRNAs were resistant to nuclease degradation.[37−39] These cholesterol-conjugated siRNAs exhibited RNAi activity without any transfection reagent but only at high (micromolar) concentrations. Therefore, it is appreciated that cholesterol-conjugated siRNAs are candidates for RNAi molecules with the potential to silence genes *in vivo*. In this study, we investigated whether Lo25D-C16/27RNA or Lo25-Chol/27RNA would be suitable for the next generation of RNAi molecules. Lo25-Chol/27RNA was a substrate of Dicer that led to the release of a 21-nt siRNA. The high RNAi potencies of Lo25-Chol/27RNA to target to *Renilla* luciferase were also verified in both the presence and absence of LF2000. However, Lo25D-C16/27RNA was determined to have higher RNAi potency than Lo25D-Chol/27RNA in HeLa cells in the presence or absence of LF2000. Although the literature describes excellent RNAi potency of the cholesterol-conjugated siRNAs both *in vitro* and *in vivo*,[37] our data suggested that Lo25D-C16/27RNA used in this study, rather than cholesterol-conjugated siRNAs, is certain to become a new generation of RNAi therapeutic agent. These intensified inhibitory effects of Lo25D-C16/27RNA are attributable to their high stability against nuclease degradation. Another report also described that lipid-conjugated siRNAs survived to a much greater extent than nonmodified siRNAs as a result of their interaction with lipoprotein particles.[24] Although efficient cellular uptake of FAM-labeled Lo25D-Chol/27RNA was observed in HeLa in the presence of LF2000, the HeLa cells treated with FAM-labeled Lo25D-C16/27RNA exhibited bright fluorescence in the cells. The FACS analysis of the HeLa cells treated with Lo25D-C16/27RNA also revealed high membrane permeability compared with that of Lo25D-Chol/27RNA. This efficient membrane permeability of Lo25D-C16/27RNA is attributable to its satisfactory interaction with both the cellular membrane and palmitic acid. These results suggested that the properties of efficient cellular uptake were among the reasons why Lo25D-C16/27RNA enhanced RNAi efficacy.

In conclusion, most of the LoRNAs and C16-LoRNAs exhibited stronger gene-silencing activity than siRNAs and C16-siRNAs. Especially, Lo25D-C16/27RNA showed excellent gene-silencing properties to promote RNAi mechanisms, by virtue of its enhanced membrane permeability and accelerated RISC selection through Dicer cleavage. Although Lo25D-Chol/27RNA showed excellent properties as an RNAi molecule, Lo25D-C16/27RNA excelled in all things: nuclease resistance, cell membrane permeability, and RNAi efficacy. We are certain that Lo25D-C16/27RNA can be useful as a new generation of RNAi molecule to overcome some of the limitations of RNAi technology and that it is applicable to various biotechnologies, including *in vivo* applications.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-82-878-9447. Fax: +81-82-878-9540. E-mail: kubo-t@yasuda-u.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported in part by The Science Research Promotion Fund from The Promotion and Mutual Aid Corporation for Private Schools of Japan. We thank Dr. Yoshifumi Takei for suggestions on how to improve the manuscript.

■ REFERENCES

(1) Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature 391*, 806−811.

(2) Kim, D., and Rossi, J. J. (2008) RNAi mechanisms and applications. *Biotechniques 44*, 613−616.

(3) John, M., Constien, R., Akinc, A., Goldberg, M., Moon, Y. A., Spranger, M., Hadwiger, P., Soutschek, J., Vornlocher, H. P., Manoharan, M., Stoffel, M., Langer, R., Anderson, D. G., Horton, J. D., Koteliansky, V., and Bumcrot, D. (2007) Effective RNAi-mediated gene silencing without interruption of the endogenous microRNA pathway. *Nature 449*, 745−747.

(4) Geisbert, T. W., Lee, A. C., Robbins, M., Geisbert, J. B., Honko, A. N., Sood, V., Johnson, J. C., Jong, S., Tavakoli, I., Judge, A., Hensley, L. E., and MacLachlan, I. (2010) Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet 375*, 1896−1905.

(5) Pan, X., Thompson, R., Meng, X., Wu, D., and Xu, L. (2011) Tumor-targeted RNA-interference: functional non-viral nanovectors. *Am. J. Cancer Res. 1*, 25−42.
(6) Myers, J. W., Jones, J. T., Meyer, T., and Ferrell, J. E. (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nat. Biotechnol. 21*, 324−328.
(7) Macrae, I. J., Zhou, K., Li, F., Repic, A., Brooks, A. N., Cande, W. Z., Adams, P. D., and Doudna, J. A. (2006) Structural basis for double-stranded RNA processing by Dicer. *Science 311*, 195−198.
(8) Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M., and Burgyan, J. (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J. 21*, 3070−3080.
(9) Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., Fougerolles, A., Endres, S., and Hartmann, G. (2005) Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med. 11*, 263−270.
(10) Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411*, 494−498.
(11) Schütze, N. (2004) siRNA technology. *Mol. Cell. Endocrinol. 213*, 115−119.
(12) Leung, R. K., and Whittaker, P. A. (2005) RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacol. Ther. 107*, 222−239.
(13) Braasch, D. A., Jensen, S., Liu, T., Kaur, K., Arar, K., White, M. A., and Corey, D. R. (2003) RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry 42*, 7967−7975.
(14) Sipa, K., Sochacka, E., Kazmierczak-Baranska, J., Maszewska, M., Janicka, M., Nowak, G., and Nawrot, B. (2007) Effect of base modifications on structure, thermodynamic stability, and gene silencing activity of short interfering RNA. *RNA 13*, 1301−1316.
(15) Chiu, Y. L., and Rana, T. M. (2003) siRNA function in RNAi: A chemical modification analysis. *RNA 9*, 1034−1048.
(16) Corey, D. R. (2007) Chemical modification: the key to clinical application of RNA interference? *J. Clin. Invest. 117*, 3615−3622.
(17) Snøve, O., Jr., and Rossi, J. J. (2006) Chemical modifications rescue off-target effects of RNAi. *ACS Chem. Biol. 1*, 274−276.
(18) Kim, D. H., Behlke, M. A., Rose, S. D., Chang, M. S., Choi, S., and Rossi, J. J. (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat. Biotechnol. 23*, 222−226.
(19) Marques, J. T., Devosse, T., Wang, D., Zamanian-Daryoush, M., Serbinowski, P., Hartmann, R., Fujita, T., Behlke, M. A., and Williams, B. R. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat. Biotechnol. 24*, 559−565.
(20) Kubo, T., Zhelev, Z., Ohba, H., and Bakalova, R. (2007) Modified 27-nt dsRNAs with dramatically enhanced stability in serum and long-term RNAi activity. *Oligonucleotides 17*, 445−464.
(21) Kubo, T., Zhelev, Z., Bakalova, R., and Ohba, H. (2007) Enhancement of gene silencing potency and nuclease stability by chemically modified duplex RNA. *Nucleic Acids Symp. Ser. 51*, 407−408.
(22) Kubo, T., Zhelev, Z., Bakalova, R., and Ohba, H. (2008) Highly efficient gene suppression by chemically modified 27 nucleotide double-stranded RNAs. *Jpn. J. Appl. Phys. 47*, 1346−1350.
(23) Rose, S. D., Kim, D. H., Amarzguioui, M., Heidel, J. D., Collingwood, M. A., Davis, M. E., Rossi, J. J., and Behlke, M. A. (2005) Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res. 33*, 4140−4156.
(24) Amarzguioui, M., Lundberg, P., Cantin, E., Hagstrom, J., Behlke, M. A., and Rossi, J. J. (2006) Rational design and *in vitro* and *in vivo* delivery of Dicer substrate siRNA. *Nat. Protoc. 1*, 508−517.
(25) Amarzguioui, M., Rossi, J. J., and Kim, D. (2005) Approaches for chemically synthesized siRNA and vector-mediated RNAi. *FEBS Lett. 579*, 5974−5981.
(26) Sano, M., Sierant, M., Miyagishi, M., Nakanishi, M., Takagi, Y., and Sutou, S. (2008) Effect of asymmetric terminal structures of short RNA duplexes on the RNA interference activity and strand selection. *Nucleic Acids Res. 36*, 5812−5821.
(27) Sun, X., Rogoff, H. A., and Li, C. J. (2008) Asymmetric RNA duplexes mediate RNA interference in mammalian cells. *Nat. Biotechnol. 26*, 1379−1382.
(28) Chu, C. Y., and Rana, T. M. (2008) Potent RNAi by short RNA triggers. *RNA 14*, 1714−1719.
(29) Ding, H., Liao, G., Wang, H., and Zhou, Y. (2007) Asymmetrically designed siRNAs and shRNAs enhance the strand specificity and efficacy in RNAi. *J. RNAi Gene Silencing 4*, 269−280.
(30) Kubo, T., Zhelev, Z., Ohba, H., and Bakalova, R. (2008) Chemically modified symmetric and asymmetric duplex RNAs: an enhanced stability to nuclease degradation and gene silencing effect. *Biochem. Biophys. Res. Commun. 365*, 54−61.
(31) Wu, S. Y., Chen, T. M., Gmeiner, W. H., Chu, E., and Schmitz, J. C. (2013) Development of modified siRNA molecules incorporating 5-fluoro-2′-deoxyuridine residues to enhance cytotoxicity. *Nucleic Acids Res. 41*, 4650−4659.
(32) Kubo, T., Yanagihara, K., Takei, Y., Mihara, K., Sato, Y., and Seyama, T. (2012) SiRNAs conjugated with aromatic compounds induce RISC-mediated antisense strand selection and strong gene-silencing activity. *Biochem. Biophys. Res. Commun. 426*, 571−577.
(33) Kubo, T., Yanagihara, K., Sato, Y., Morita, Y., and Seyama, T. (2012) Enhancement of gene silencing effect and membrane permeability by peptide-conjugated 27-nucleotide small interfering RNA. *Molecules 17*, 11089−11102.
(34) Kubo, T., Yanagihara, K., Takei, Y., Mihara, K., Morita, Y., and Seyama, T. (2011) Palmitic acid-conjugated 21-nucleotide siRNA enhances gene-silencing activity. *Mol. Pharmaceutics 8*, 2193−2203.
(35) Kubo, T., Yanagihara, K., Takei, Y., Mihara, K., Sato, Y., and Seyama, T. (2012) Lipid-conjugated 27-nucleotide double-stranded RNAs with dicer-substrate potency enhance RNAi-mediated gene silencing. *Mol. Pharmaceutics 9*, 1374−1383.
(36) Kubo, T., Takei, Y., Mihara, K., Yanagihara, K., and Seyama, T. (2012) Amino-modified and lipid-conjugated dicer-substrate siRNA enhances RNAi efficacy. *Bioconjugate Chem. 15*, 164−173.
(37) Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, P. K., Racie, T., Rajeev, K. G., Röhl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., and Vornlocher, H. P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature 432*, 173−178.
(38) Wolfrum, C., Shi, S., Jayaprakash, K. N., Jayaraman, M., Wang, G., Pandey, R. K., Rajeev, K. G., Nakayama, T., Charrise, K., Ndungo, E. M., Zimmermann, T., Koteliansky, V., Manoharan, M., and Stoffel, M. (2007) Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. *Nat. Biotechnol. 25*, 1149−1157.
(39) Ding, Y., Wang, W., Feng, M., Wang, Y., Zhou, J., Ding, X., Zhou, X., Liu, C., Wang, R., and Zhang, Q. (2012) A biomimetic nanovector-mediated targeted cholesterol-conjugated siRNA delivery for tumor gene therapy. *Biomaterials 33*, 8893−8905.
(40) Nishina, K., Unno, T., Uno, Y., Kubodera, T., Kanouchi, T., Mizusawa, H., and Yokota, T. (2008) Efficient *in vivo* delivery of siRNA to the liver by conjugation of alpha-tocopherol. *Mol. Ther. 16*, 734−740.
(41) Wong, N., Liu, Z., and Dai, H. (2005) Functionalization of carbon nanotubes via cleavable disulfide bonds for efficient intracellular delivery of siRNA and potent gene silencing. *J. Am. Chem. Soc. 127*, 12492−12493.
(42) MacRae, I. J., Zhou, K., and Doudna, J. A. (2007) Structural determinants of RNA recognition and cleavage by Dicer. *Nat. Struct. Mol. Biol. 14*, 934−940.
(43) Vermeulen, A., Behlen, L., Reynolds, A., Wolfson, A., Marshall, W. S., Karpilow, J., and Khvorova, A. (2005) The contributions of dsRNA structure to Dicer specificity and efficiency. *RNA 11*, 674−682.
(44) Ameres, S. L., Martinez, J., and Schroeder, R. (2007) Molecular basis for target RNA recognition and cleavage by human RISC. *Cell 130*, 101−112.

(45) Lima, W. F., Prakash, T. P., Murray, H. M., Kinberger, G. A., Li, W., Chappell, A. E., Li, C. S., Murray, S. F., Gaus, H., Seth, P. P., Swayze, E. E., and Crooke, S. T. (2012) Single-stranded siRNAs activate RNAi in animals. *Cell 150,* 883−894.