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Synthesis and silencing properties of siRNAs possessing lipophilic groups at their 3′-termini

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ABSTRACT

Short-interfering RNAs (siRNAs) conjugated with lipophilic groups at their 3′-termini were synthesized. The properties of the synthesized siRNAs were examined in detail, and it was found that at low concentrations, their silencing abilities were dependent on the positions of the modifications and the types of organic molecules attached. Although the modification of siRNAs with palmitic acid or oleic acid at the 3′-end slightly reduced their silencing activities, siRNAs had enough abilities to induce RNAi at 10 nM concentrations. On the other hand, the modification of siRNAs with cholesterol at the 3′-end of the passenger strand was tolerated; however, the modification at the guide strand significantly reduces its silencing activity. The siRNAs modified with the lipophilic groups did not possess ability to penetrate the plasma membranes of HT-1080 cells without the transfection reagent. However, the results described in this report will aid in designing novel siRNAs with cell membrane-permeable molecules.

1. Introduction

Since the discovery of RNA interference (RNAi) as a means to silence expression of specific genes, small-interfering RNAs (siRNAs) have attracted significant attention as powerful tools for targeting therapeutically important mRNAs and eliciting their cleavage.[1,2] siRNA has considerable potential as a new therapeutic drug for intractable diseases because siRNAs can be logically designed and synthesized if the sequences of disease-causing genes are known. One of the critical problems in RNAi-based therapeutic applications is the delivery of siRNA across plasma membranes of cells in vivo. Thus far, a number of solutions to this problem have been reported.[3–9] Among them, the direct chemical modification of siRNAs is an attractive proposition because various kinds of organic molecules, which are specifically uptaken in specific tissues, can be specifically introduced into siRNAs. Recently, Soutschek et al. have succeeded in reducing plasma low-density lipoprotein (LDL) levels in mice by using siRNAs that are modified with cholesterol at the 3′-end of the guide strand (antisense strand).[4]

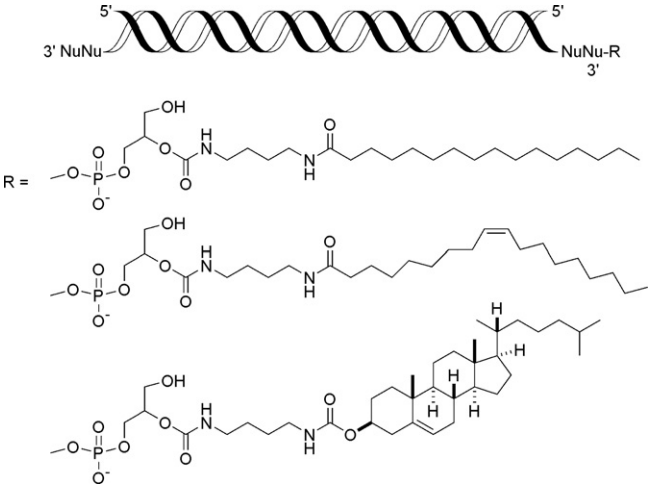

Figure 1. Structures of siRNAs possessing lipophilic groups.

It is deduced that the ability of siRNAs to induce RNAi depends on the positions of modifications and kinds of organic molecules attached. However, the silencing activities of siRNAs modified with

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cell membrane-permeable molecules have not been investigated systematically. In this paper, we report the synthesis and silencing properties of siRNAs possessing lipophilic groups at their 3′-termini (Fig. 1).

Argonaute2, a key component of RNA-induced silencing complex (RISC), is responsible for mRNA cleavage in the RNAi pathway.[10] Argonaute2 is composed of PAZ, Mid, and PIWI domains. The X-ray structural analysis of a co-crystal between a PIWI protein and an siRNA reveals that the 5′-phosphate of a guide strand (antisense strand) of siRNA is recognized by the PIWI domain and that the 5′-phosphate interacts with the carboxyl group of a C-terminal residue of the domain through a divalent metal ion.[11,12] It is observed that the modification of the 5′-hydroxyl groups of the guide strands of siRNAs by methoxy groups completely hampers the functions of siRNAs to induce RNAi in experiments using *Drosophila* embryo lysate and HeLa cell extract.[13,14] These results imply that the 5′-terminus of a guide strand is not suitable for modification by lipophilic groups.

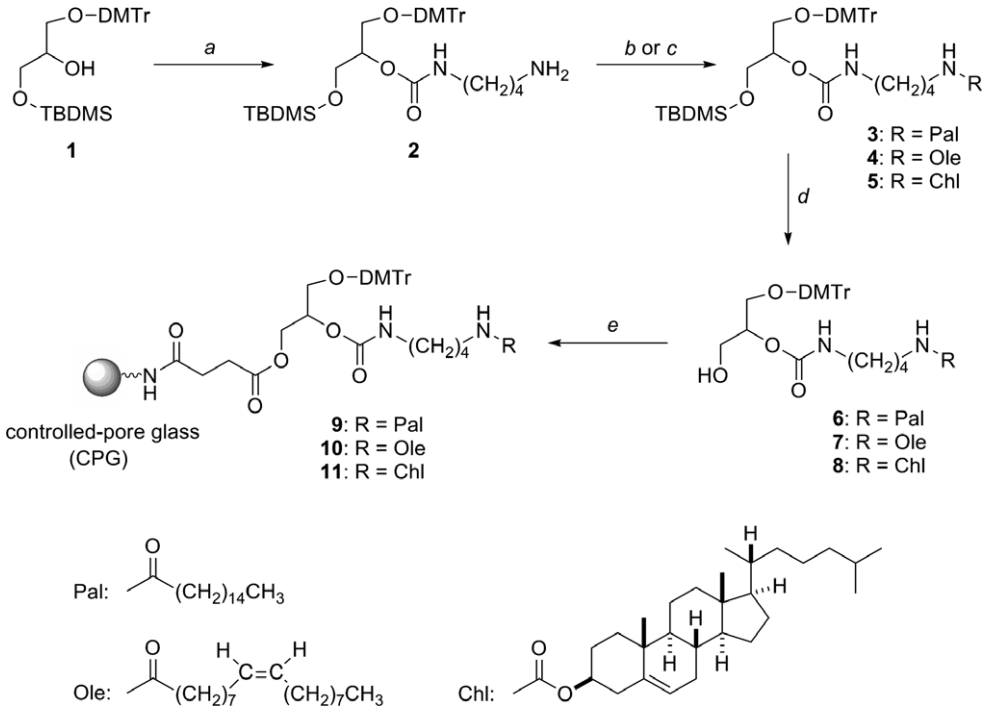
On the other hand, X-ray structural analysis and a nuclear magnetic resonance (NMR) study have revealed that the 3′-overhang region of a guide strand of siRNA is recognized by the PAZ domain and the 2-nucleotide (nt) 3′-overhang is accommodated into a binding pocket composed of hydrophobic amino acids in the domain.[15–18] The length of the 3′-overhang regions of siRNA influences the activities of siRNAs. It is reported that the 2-nt 3′-overhang is the most efficient in an experiment using 21-nt siRNA in *Drosophila* embryo lysate; however, the multiple addition of 2′-deoxynucleotide to the 3′-end of siRNAs is tolerated.[19] On the basis of this information, we introduced lipophilic groups at the 3′-ends of the passenger or guide strands of siRNAs.

2. Results and discussion

Soutschek et al. used 4-hydroxyprolinol as a linker for conjugating RNAs and lipophilic groups. We selected glycerol, which was more flexible than hydroxyprolinol, as a linker to join RNAs and lipophilic groups. Glycerol derivative **1**[20] with 4,4′-dimethoxytrityl (DMTr) and *tert*-butyldimethylsilyl (TBDMS) groups at primary hydroxyls was synthesized according to the reported method, converted into its *O*-carbonylimidazolide, and reacted with 1,4-diaminobutane to afford an aminobutylcarbamoyl derivative **2** in 94% yield (Scheme 1). Palmitic acid and oleic acid were introduced into **2** in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI) to yield glycerol derivatives **3** and **4** with fatty acids in 69% and 86% yields, respectively. On the other hand, cholesterol was converted into its *O*-carbonylimidazolide, which was then reacted with **2** to afford a cholesterol-modified glycerol **5** in 64% yield. After the desilylation of **3**, **4**, and **5** by tetrabutylammonium fluoride (TBAF), hydroxyl derivatives **6**, **7**, and **8** were succinated to give the corresponding succinates. They were linked to a controlled pore glass (CPG) to produce solid supports **9**, **10**, and **11** containing **6** (28 μmol/g), **7** (83 μmol/g), and **8** (88 μmol/g), respectively.

All oligoribonucleotides (ONs) were synthesized using solid supports **9**, **10**, and **11** by using a DNA/RNA synthesizer (Table 1). Fully protected ONs (1.0 μmol each) linked to solid supports were treated with concentrated NH_4OH/EtOH (3:1, v/v) at room temperature for 12 h and then with 1.0 M TBAF/THF at room temperature for 12 h. Released ONs were purified by denaturing 20% polyacrylamide gel electrophoresis (20% PAGE) to afford deprotected ONs **25**–**44**. These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and observed molecular weights were in agreement with their structures.

The ability of modified siRNAs to suppress gene expression was studied by a dual-luciferase assay using a psiCHECK-2 vector, which contained *Renilla* and firefly luciferase genes. Sequences of siRNAs were designed to target *Renilla* luciferase. HeLa cells were co-transfected with the vector and indicated amounts of siRNAs. Signals of *Renilla* luciferase were normalized to that of firefly



Scheme 1. Reagents and conditions: (a) (1) *N*,*N*′-carbonyldiimidazole, pyridine, rt, 1 h; (2) 1,4-diaminobutane, pyridine, rt, 3 h, 94%; (b) palmitic acid or oleic acid, WSCI, CH_2Cl_2, rt, 24 h, 69% for **3** and 64% for **4**; (c) cholesterol, *N*,*N*′-carbonyldiimidazole, pyridine, rt, 24 h, 64%; (d) TBAF, THF, rt, 80% for **6**, 82% for **7**, and 53% for **9**; (e) (1) succinic anhydride, DMAP, pyridine, rt, 24 h; (2) CPG, WSCI, DMF, rt, 72 h, 28 μmol/g for **9**, 83 μmol/g for **10**, and 88 μmol/g for **11**.

Table 1
Sequences of ONs and siRNAs used in this study

No. of siRNA	No. of ON	Sequence
siRNA**12**	ON**25**	5′-GGCCUUUCACUACUCCUAC*tt*-3′
	ON**26**	3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**13**	ON**27**	5′-GGCCUUUCACUACUCCUAC*tt*-3′-**Pal**
	ON**26**	3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**14**	ON**28**	5′-GGCCUUUCACUACUCCUAC*tt*-3′-**Ole**
	ON**26**	3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**15**	ON**29**	5′-GGCCUUUCACUACUCCUAC*tt*-3′-**Chl**
	ON**26**	3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**16**	ON**25**	5′-GGCCUUUCACUACUCCUAC*tt*-3′
	ON**30**	**Pal**-3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**17**	ON**25**	5′-GGCCUUUCACUACUCCUAC*tt*-3′
	ON**31**	**Ole**-3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**18**	ON**25**	5′-GGCCUUUCACUACUCCUAC*tt*-3′
	ON**32**	**Chl**-3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**19**	ON**33**	5′-UUCUCCGAACGUGUGCACGU*tt*-3′
	ON**34**	3′-*tt*AAGAGGCUUGCACAGUGCA-5′
siRNA**20**	ON**35**	5′-AGAUCCAUACGUCCGUGAA*tt*-3′
	ON**36**	3′-*tt*UCUAGGUAUGCAGGCACUU-5′
siRNA**21**	ON**37**	5′-GCACCGGCAGGAGAUCAUA*tt*-3′
	ON**38**	3′-*gt*CGUGGCCGUCCUCUAGUAU-5′
siRNA**22**	ON**39**	5′-GCUGUUCAAAACGAAGAUG*tt*-3′-**Pal**
	ON**40**	**Pal**-3′-*tt*CGACAAGUUUUGCUUCUAC-5′
siRNA**23**	ON**41**	5′-GCUGUUCAAAACGAAGAUG*tt*-3′-**Ole**
	ON**42**	**Ole**-3′-*tt*CGACAAGUUUUGCUUCUAC-5′
siRNA**24**	ON**43**	5′-GCUGUUCAAAACGAAGAUG*tt*-3′-**Chl**
	ON**44**	**Chl**-3′-*tt*CGACAAGUUUUGCUUCUAC-5′

The capital letters indicate ribonucleosides. Small italic letters represent 2′-deoxyribonucleosides.

luciferase. The silencing activities of siRNAs correlated with the positions of the modifications and kinds of lipophilic groups attached at the 3′-termini of siRNAs (Fig. 2a and b). Although the modifications of passenger strands (sense strands) by palmitic acid and oleic acid reduced the silencing activities of siRNAs by a small amount as compared to that of unmodified siRNA, siRNAs modified with these fatty acids maintained their abilities to down-regulate protein expression levels to 30% at 10 nM concentrations. The silencing activity of siRNA modified with cholesterol at the 3′-end of the passenger strand was weaker than those of siRNAs modified with palmitic acid and oleic acid at each concentration. However, it down-regulated protein expression level to approximately 50% at 10 nM concentrations. The tendency of the silencing activities of siRNAs modified with palmitic acid and oleic acid at the 3′-ends of guide strands was similar to that of siRNAs modified at the 3′-ends of guide strands. On the other hand, the modification of siRNA with cholesterol at the 3′-end of the guide strand significantly reduced its silencing activity.

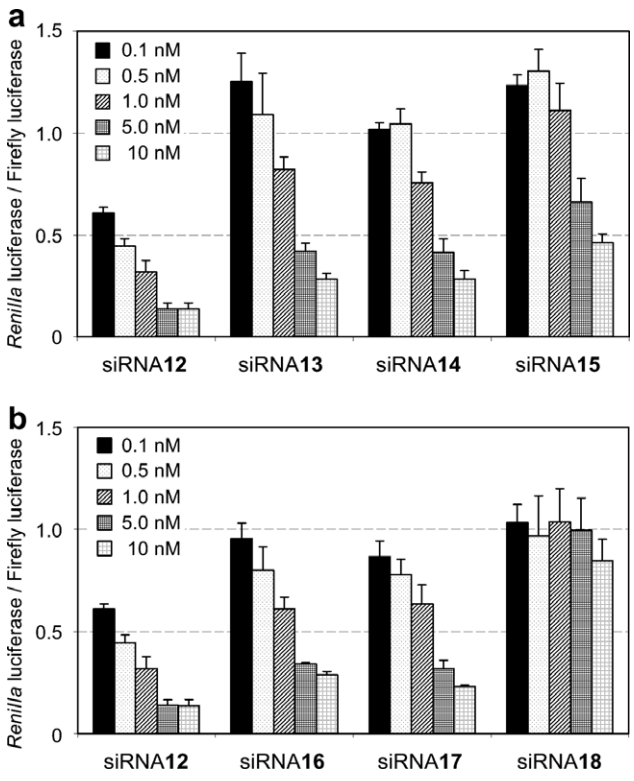


Figure 2. Dual-luciferase assay. (a) siRNAs modified at 3′-ends of passenger (sense) strands. (b) siRNAs modified at 3′-ends of guide (antisense) strands.

Argonaute2/eIF2C2 (hAgo2) has been identified as a key protein with an endonuclease activity associated with RISC in the RNAi pathway.[21,22] In order to examine whether or not the observed silencing activities could be attributed to RNAi, the activities of modified siRNAs were studied after treating HeLa cells with eIF2C2-targeting siRNAs. We considered that if the silencing activities of modified siRNAs resulted from RNAi, the expression levels of luciferase proteins would recover by treating HeLa cells with siRNAs targeting eIF2C2. Two kinds of siRNAs targeting eIF2C2—one (siRNA**20**) targeted open reading frame (ORP) positions 1168–1188 and the other (siRNA**21**) targeted ORP positions 1897–1917—were used in this study. The siRNA**19** is composed of a random sequence. HeLa cells were transfected with the siRNA**19**, **20** or **21**. After incubating them for 1 h, the cells were co-transfected with a psiCHECK-2 vector and siRNAs modified with lipophilic groups. After incubating for 24 h, the activities of *Renilla* luciferase were measured. The siRNA**19** composed of a random sequence did not largely change the expression level of *Renilla* luciferase (Fig. 3) whereas the signals of *Renilla* luciferase were recovered by treating them with siRNAs targeting eIF2C2 (Fig. 4). These results indicated that the silencing activities of modified siRNAs were attributed to RNAi.

In order to examine whether or not the siRNAs modified with the lipophilic groups can cross plasma membranes of cells without a transfection reagent, next, we performed an experiment targeting an endogenous protein. We chose an mRNA of a human RNase L protein, which is a constituent of the 2-5A system, as a target. It

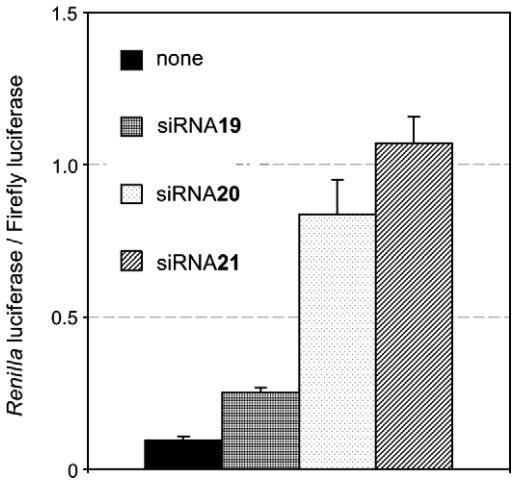


Figure 3. Dual-luciferase assay. HeLa cells were transfected with siRNA**19** (50 nM), siRNA**20** (50 nM), or siRNA**21** (50 nM). After incubating for 1 h, the cells were co-transfected with a psiCHECK-2 vector and siRNA**12** (10 nM). After incubating for 24 h, the activities of *Renilla* luciferase were measured.

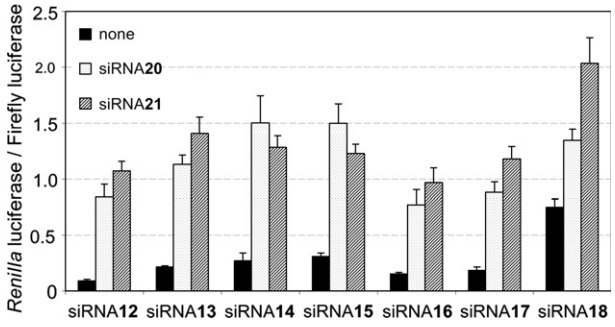


Figure 4. Dual-luciferase assay. HeLa cells were transfected with siRNA**20** (50 nM) or siRNA**21** (50 nM). After incubating for 1 h, the cells were co-transfected with a psiCHECK-2 vector and siRNAs (10 nM) modified with lipophilic groups. After incubating for 24 h, the activities of *Renilla* luciferase were measured.

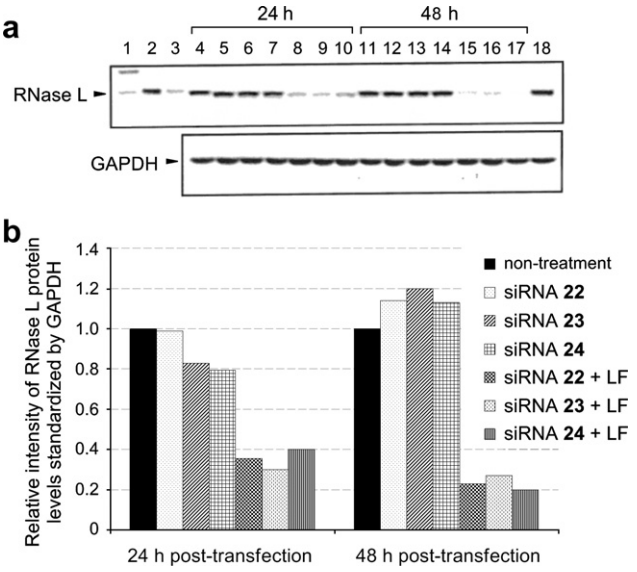


Figure 5. (a) Western blot analysis for human RNase L and GAPDH. Lanes 5–7 and 12–14, without Lipofectamine 2000; lanes 8–10 and 15–17, with Lipofectamine 2000. Lane 1, marker; lane 2, 3.5 ng RNase L; lane 3, 1.75 ng RNase L; lanes 4 and 11, non-treatment; lanes 5, 8, 12, and 15, siRNA **22**; lanes 6, 9, 13, and 16, siRNA**23**; lanes 5, 8, 12, and 15, siRNA**22**; lanes 7, 10, 14, and 17, siRNA**24**; lane 18, cells + 1.75 ng RNase L. (b) Relative intensities of RNase L protein levels standardized by GAPDH. LF indicates Lipofectamine 2000.

was reported that the absence of RNase L in vivo causes both a deficiency in the antiviral activity of interferon and a major defect in apoptosis[23,24]. We selected 23-nt of the open reading frame (ORP) position 94–112, that starts with 5′-AA-3′ and ends with 5′-TT-3′, as a target sequence. Sequences of the siRNAs are listed in Table 1. The siRNAs **22**, **23**, and **24** have palmitic acid, oleic acid, and cholesterol at the 3′-ends both of the passenger and guide strands, respectively.

HT-1080 human fibrosarcoma (HT-1080) cells were transfected with the siRNAs (200 nM concentrations) with and without the transfection reagent, and the cell lysates were analyzed 24 and 48 h later by immunoblot analysis with RNase L-specific antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein level served as a loading control. The results of the immunoblot analysis are represented in Figure 5a. Figure 5b shows the relative intensities of RNase L protein levels standardized by GAPDH.

When the cells were transfected with the siRNAs **22**, **23**, and **24** by using the transfection reagent, the human RNase L protein expression levels were reduced to ~30% and ~20% at 24 and 48 h post-transfections, respectively. Thus, it was found that the modified siRNAs had the abilities to down-regulate the expression of the target protein. On the other hand, when the cells were treated with the abovementioned siRNAs without the transfection reagent, the expression levels of the human RNase L protein hardly changed at both 24 and 48 h post-transfections. From these results, it was revealed that the siRNAs modified with palmitic acid, oleic acid, and cholesterol did not have abilities to penetrate the plasma membranes of HT-1080 cells without the transfection reagent.

In conclusion, we have demonstrated the synthesis of siRNAs possessing lipophilic groups at their 3′-termini. It was found that all the modified siRNAs had similar silencing abilities at high siRNA concentrations but the silencing abilities of the modified siRNAs at low siRNA concentrations were dependent on the kind and positions of the modifications. Although the modifications of siRNAs with palmitic acid or oleic acid at their 3′-end reduced their silencing activities by a small amount, siRNAs had enough abilities to induce RNAi at their 10 nM concentrations. On the other hand, the modification of siRNA with cholesterol at the 3′-end of the passenger strand was tolerated; however, the modification at the guide strand significantly reduces its silencing activity. From these results, it is concluded that the 3′-end of the passenger strand is better than the guide strand for conjugating bulky molecules such as fatty acids, particularly rigid molecules such as cholesterol. The siRNAs modified with the lipophilic groups did not have abilities to penetrate the plasma membranes of HT-1080 cells without the transfection reagent. However, we believe that the results described here will aid in designing novel siRNAs with cell-membrane-permeable molecules.

3. Experimental

3.1. General remarks

NMR spectra were recorded at 400 MHz (^{1}H) and at 100 MHz (^{13}C), and are reported in parts per million downfield from tetramethylsilane. Coupling constants (*J*) are expressed in Hertz. Mass spectra were obtained by fast atom bombardment (FAB). Thin-layer chromatography was carried out on Merck-coated plates 60 F_{254}. Silica gel column chromatography was carried out on Wako gel C-300. siRNAs directed against eIF2C2 (hAgo2) were purchased from Qiagen Inc.

3.1.1.1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-(4-aminobutyl)carbamoyl]-3-*O*-tert-butyldimethylsilylglycerol (2)

A mixture of 1-*O*-(4,4′-dimethoxytrityl)-3-*O*-*tert*-butyldimethylsilylglycerol (**1**) (2.60 g, 5.11 mmol) and *N*,*N*′-carbonyldiimidazole (1.66 g, 10.2 mmol) in pyridine (17 mL) was stirred at room temperature. After 1 h, 1,4-diaminobutane (2.63 g, 29.8 mmol) was added to the mixture at 0 °C, and the whole was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$. The organic layer was washed with H_2O and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 10–25% MeOH in $CHCl_3$) to give **2** (3.00 g, 4.82 mmol) in 94% yield: ^{1}H NMR (DMSO-d_6) δ 0.13 (s, 6H), 0.97 (s, 9H), 3.94 (s, 6H), 6.96–7.61 (m, 13H), all additional signals correspond to glycerol and aminobutyl moieties; ^{13}C NMR (DMSO-d_6) δ *−5.6*, 17.7, 25.6, 26.8, 27.8, 48.5, 55.0, 61.7, 62.3, 72.5, 85.2, 113.1, 126.6, 127.6, 127.7, 129.6, 135.5, 144.9, 149.3, 155.8, 158.0; HRMS (FAB) calcd for $C_{35}H_{51}N_2O_6Si$ (MH^+) 623.3516, found 623.3508.

3.1.2. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-palmitoyl-N-(4-aminobutyl)-carbamoyl]-3-*O*-*tert*-butyldimethylsilylglycerol (3)

A mixture of **2** (0.90 g, 1.44 mmol), palmitic acid (0.55 g, 2.16 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI) in CH_2Cl_2 (14 mL) was stirred at room

temperature. After 24 h, the mixture was diluted with $CHCl_3$. The organic layer was washed with H_2O and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 10–50% EtOAc in hexane) to give **3** (0.85 g, 0.99 mmol) in 69% yield: ^{1}H NMR ($CDCl_3$) δ 0.01 (s, 6H), 0.82 (s, 9H), 3.80 (s, 6H), 6.81–7.46 (m, 13H), all additional signals correspond to glycerol, palmitoyl, and aminobutyl moieties; ^{13}C NMR ($CDCl_3$) δ *−5.4,* 14.1, 18.1, 22.7, 25.7, 26.6, 27.5, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 36.7, 39.0, 40.3, 55.1, 60.4, 62.1, 62.4, 74.0, 85.7, 113.0, 126.6, 127.7, 128.1, 130.0, 130.1, 136.0, 136.1, 144.9, 156.2, 158.3, 173.3.

3.1.3. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-palmitoyl-N-(4-aminobutyl)carbamoyl]glycerol (6)

A mixture of **3** (0.75 g, 0.87 mmol) and TBAF (1 M in THF, 2.00 mL, 2.00 mmol) in THF (4.4 mL) was stirred at room temperature. After 1 h, the mixture was diluted with EtOAc. The organic layer was washed with H_2O and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, EtOAc) to give **6** (0.52 g, 0.70 mmol) in 80% yield: ^{1}H NMR ($CDCl_3$) δ 3.81 (m, 6H), 6.81–7.43 (m, 13H), all additional signals correspond to glycerol, palmitoyl and aminobutyl moieties; ^{13}C NMR ($CDCl_3$) δ 13.8, 14.1, 20.5, 22.7, 25.7, 26.7, 27.2, 29.3, 29.5, 29.6, 29.7, 31.9, 36.8, 39.0, 40.6, 52.1, 55.2, 62.9, 63.2, 74.6, 86.1, 113.1, 126.8, 127.8, 128.0, 130.0, 135.7, 144.6, 156.6, 158.4, 173.4; HRMS (FAB) calcd for $C_{45}H_{67}N_2O_7$ (MH^+) 747.4949, found 747.4955.

3.1.4. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-oleoyl-N-(4-aminobutyl)carbamoyl]-3-*O*-*tert*-butyldimethylsilylglycerol (4)

A mixture of **2** (1.50 g, 2.41 mmol), oleic acid (1.15 mL, 3.62 mmol), and WSCI in CH_2Cl_2 (12 mL) was stirred at room temperature. After 24 h, the mixture was diluted with $CHCl_3$. The organic layer was washed with H_2O and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 0–5% MeOH in $CHCl_3$) to give **4** (1.83 g, 2.06 mmol) in 86% yield: ^{1}H NMR ($CDCl_3$) δ 0.04 (s, 6H), 0.85 (s, 9H), 3.82 (s, 6H), 6.84–7.49 (m, 13H), all additional signals correspond to glycerol, oleoyl, and aminobutyl moieties; ^{13}C NMR ($CDCl_3$) δ *−5.4,* 14.1, 18.1, 22.7, 25.7, 26.6, 27.1, 27.2, 27.5, 29.1, 29.2, 29.3, 29.5, 29.6, 29.7, 29.8, 31.9, 36.7, 39.0, 40.4, 50.6, 55.1, 60.4, 62.2, 62.4, 74.0, 85.8, 113.0, 126.6, 127.7, 128.1, 129.7, 130.0, 130.1, 136.0, 136.1, 144.9, 156.3, 158.4, 173.3; Anal. Calcd for $C_{53}H_{82}N_2O_7Si{\cdot}CHCl_3$: C, 64.43; H, 8.31; N, 2.78. Found: C, 64.32; H, 8.33; N, 2.75.

3.1.5. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-oleoyl-*N*-(4-aminobutyl)carbamoyl]glycerol (7)

A mixture of **4** (1.66 g, 1.88 mmol) and TBAF (1 M in THF, 3.76 mL, 3.76 mmol) in THF (18 mL) was stirred at room temperature. After 7 h, the mixture was diluted with EtOAc. The organic layer was washed with H_2O and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 0–10% MeOH in $CHCl_3$) to give **7** (1.19 g, 1.54 mmol) in 82% yield: ^{1}H NMR ($CDCl_3$) δ 3.78 (s, 6H), 6.81–7.43 (m, 13H), all additional signals correspond to glycerol, oleoyl, and aminobutyl moieties; ^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 25.7, 26.7, 27.1, 29.1, 29.2, 29.4, 29.6, 29.7, 31.8, 32.5, 36.7, 38.9, 40.5, 55.1, 62.8, 63.1, 74.5, 86.1, 113.1, 126.7, 127.8, 128.0, 129.7, 129.9, 135.7, 144.6, 156.6, 158.4, 173.3; Anal. Calcd for $C_{47}H_{68}N_2O_7{\cdot}10/3CHCl_3$: C, 51.63; H, 6.14; N, 2.93. Found: C, 51.79; H, 6.08; N, 2.48.

3.1.6. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-cholesteryloxycarbonyl-*N*-(4-aminobutyl)carbamoyl]-3-*O*-*tert*-butyldimethylsilylglycerol (5)

A mixture of cholesterol (0.22 g, 0.58 mmol) and *N*,*N*′-carbonyldiimidazole (0.10 g, 0.63 mmol) in pyridine (3 mL) was stirred at room temperature. After 2 h, **2** (0.43 g, 0.70 mmol) was added to the mixture, and the whole was stirred at room temperature. After 12 h, the mixture was diluted with EtOAc. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 10% EtOAc in hexane) to give **5** (0.39 g, 0.37 mmol) in 64% yield: ^{1}H NMR ($CDCl_3$) δ 0.10 (s, 6H), 0.81 (s, 9H), 3.79 (s, 6H), 6.80–7.44 (m, 13H), all additional signals correspond to glycerol, cholesterol, and aminobutyl moieties; ^{13}C NMR ($CDCl_3$) δ *−5.4, −3.6,* 11.8, 18.0, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.3, 25.6, 25.8, 27.3, 28.0, 28.1, 28.2, 31.8, 31.9, 35.8, 36.2, 36.5, 37.0, 38.5, 39.5, 39.7, 40.6, 42.3, 50.0, 55.2, 56.1, 56.7, 62.1, 85.8, 113.0, 122.4, 126.6, 127.7, 128.1, 130.0, 136.1, 139.8, 144.9, 156.1, 158.3; Anal. Calcd for $C_{63}H_{94}N_2O_8Si{\cdot}2H_2O$: C, 70.62; H, 9.22; N, 2.61. Found: C, 70.72; H, 9.45; N, 2.43.

3.1.7. 1-*O*-(4,4′-Dimethoxytrityl)-2-*O*-[*N*-cholesteryloxycarbonyl-*N*-(4-aminobutyl)carbamoyl]glycerol (8)

A mixture of **5** (0.34 g, 0.33 mmol) and TBAF (1 M in THF, 1.00 mL, 1.00 mmol) in THF (5 mL) was stirred at room temperature. After 4 h, the mixture was concentrated in vacuo. The residue was purified by column chromatography (SiO_2, 20–100% EtOAc in hexane) to give **8** (0.61 g, 0.18 mmol) in 53% yield: ^{1}H NMR ($CDCl_3$) δ 3.73 (s, 6 H), 6.75–7.37 (m, 13H), all additional signals correspond to glycerol, cholesterol, and aminobutyl moieties; ^{13}C NMR ($CDCl_3$) δ 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.3, 27.1, 27.3, 28.0, 28.1, 28.2, 31.8, 31.9, 35.8, 36.1, 36.5, 36.9, 38.5, 39.5, 39.7, 40.7, 42.3, 49.9, 55.2, 56.1, 56.6, 62.9, 63.4, 74.5, 86.2, 113.1, 122.5, 126.8, 127.8, 128.0, 130.0, 130.1, 135.7, 135.7, 139.8, 144.6, 156.2, 158.5; Anal. Calcd for $C_{57}H_{80}N_2O_8{\cdot}1/3H_2O$: C, 73.83; H, 8.77; N, 3.02. Found: C, 73.87; H, 8.68; N, 2.92.

3.2. Solid support synthesis

A mixture of **6** (0.52 g, 0.70 mmol), succinic anhydride (0.21 g, 2.10 mmol), and DMAP (86 mg, 0.70 mmol) in pyridine (7 mL) was stirred at room temperature. After 24 h, the solution was partitioned between $CHCl_3$ and H_2O, and the organic layer was washed with H_2O and brine. The separated organic phase was dried (Na_2SO_4) and concentrated to give a succinate. Aminopropyl controlled pore glass (0.81 g, 0.18 mmol) was added to a solution of the succinate (0.55 g, 0.61 mmol) and WSCI (0.13 g, 0.70 mmol) in DMF (18 mL), and the mixture was kept for 72 h at room temperature. After the resin was washed with pyridine, a capping solution (20 mL, 0.1 M DMAP in pyridine/Ac_2O = 9:1, v/v) was added and the whole mixture was kept for 24 h at room temperature. The resin was washed with MeOH and acetone, and dried in vacuo. Amount of loaded compound **6** to solid support was 28 μmol/g from calculation of released dimethoxytrityl cation by a solution of 70% $HClO_4$/EtOH (3:2, v/v). In a similar manner, solid supports with **7** and **8** were obtained in 83 and 88 μmol/g loading amounts, respectively.

3.3. RNA synthesis

Synthesis was carried out with a DNA/RNA synthesizer by phosphoramidite method. Deprotection of bases and phosphates was performed in concentrated NH_4OH/EtOH (3:1, v/v) at room temperature for 12 h. 2′-TBDMS groups were removed by 1.0 M tetrabutylammonium fluoride (TBAF, Aldrich) in THF at room temperature for 12 h. The reaction was quenched with 0.1 M TEAA buffer (pH 7.0) and desalted on a Sep-Pak C18 cartridge. Deprotected ONs were purified by 20% PAGE containing 7 M urea to give the highly purified ON**27** (49), ON**28** (24), ON**29** (7), ON**30** (41), ON**31** (19), ON**32** (11), ON**39**(13), ON**40**(8), ON**41**(9), ON**42**(9), ON**43**(11), and ON**44**(6). The yields are indicated in parentheses as OD units at 260 nm starting from 1.0 μmol scale. Extinction coefficients of the

ONs were calculated from those of mononucleotides and dinucleotides according to the nearest-neighbor approximation method.[25]

3.4. MALDI-TOF/MS analyses of RNAs

Spectra were obtained with a time-of-flight mass spectrometer. ON**27**: calculated mass, 7009.5; observed mass, 7003.4. ON**28**: calculated mass, 7035.5; observed mass, 7025.8. ON**29**: calculated mass, 7183.8; observed mass, 7177.2. ON**30**: calculated mass, 7318.8; observed mass, 7311.9. ON**31**: calculated mass, 7344.8; observed mass, 7337.4. ON**32**: calculated mass, 7493.0; observed mass, 7486.3. ON**39**: calculated mass, 7223.7; observed mass, 7220.4. ON**40**: calculated mass, 7074.5; observed mass, 7070.1. ON**41**: calculated mass, 7249.8; observed mass, 7250.2. ON**42**: calculated mass, 7100.6; observed mass, 7099.4. ON**43**: calculated mass, 7398.0; observed mass, 7397.8. ON44: calculated mass, 6530.0; observed mass, 6538.4.

3.5. Dual-luciferase assay

HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours before transfection, HeLa cells (4×10^4/mL) were transferred to 96-well plates (100 μL per well). They were transfected, using TransFast (Promega), according to instructions for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psiCHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I Reduced-Serum Medium (Invitrogen), and incubated at 37 °C. After 1 h, MEM (100 μL) containing 10% FBS and antibiotics was added to each well, and the whole was further incubated at 37 °C. After 24 h, cell extracts were prepared in Passive Lysis Buffer (Promega). Activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (Promega) according to the manufacturer's protocol. The results were confirmed by at least three independent transfection experiments with two cultures each and are expressed as the average from four experiments as means ± SD.

3.6. Cell culture and transfection

HT-1080 human fibrosarcoma (HT-1080) cells were cultured in RPMI 1640 supplemented with streptomycin (0.1 mg/mL), penicillin (100 U/mL), and 10% heat-inactivated fetal bovine serum. Cells were cultured at 37 °C with a 5% CO_2 atmosphere. Cells were seeded in 35-mm dishes at density of 120,000 cells per dish and allowed to attach to the culture vessel for 24 h prior to transfection. Transfection solutions were prepared according to the manufacturer's protocols with an antibiotic-free medium. siRNA was diluted in 250 μL of RPMI 1640 without antibiotics and serum. Five micrograms of Lipofectamine 2000 (Invitrogen) was diluted in 250 μl of RPMI 1640 without antibiotics and serum. After 5 min of incubation, they were combined and incubated for 20 min at room temperature to form the siRNA-lipid complexes. Five hundred microliters of the transfection solution was dispensed per dish. Transfections were conducted for 6 h, after which the solution was removed and replaced with growth medium containing antibiotics. Cells were harvested for each analysis 24 or 48 h post-transfection.

3.7. Western blot analysis for human RNase L

HT-1080 cells were harvested by washing the cells once with 1× PBS, aspirated, and treated with 1 mL of a trypsin solution at 37 °C for 1 min. Trypsin was inactivated by the addition of a culture medium containing 10% FBS. The contents of each dish were transferred separately into 1.5 mL microfuge tubes and centrifuged at 2500 rpm for 5 min at 4 °C. The supernatant fluid was discarded, and the cell pellet was resuspended in 2 volumes of hypotonic buffer A (0.5% (v/v) Nonidet P-40, 20 mM Hepes, pH 7.5, 10 mM KOAc, 15 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin) and allowed to swell in this buffer for 10 min before being broken either by 30 strokes in a tight-fitting glass Dounce homogenizer for 2 min in ice. The homogenate was centrifuged at 10,000g for 10 min and the supernatant was pipetted off and stored at −80 °C. Cell extracts were separated by 7.5% SDS–PAGE. The proteins were electrophoretically transferred to PVDF membrane. Primary antibodies, mouse monoclonal anti-human RNase L antibody, that were obtained from Taiho Pharmaceutical Co., Ltd, were diluted 1:5600 in TTBS containing 5% BSA and placed on a rocker platform for 16 h at 4 °C. The membranes were washed once for 15 min and then three times for 5 min each in TTBST. Secondary antibody conjugate (HRP conjugated anti-mouse IgG) was diluted 1:100,000 in TTBS containing 5% BSA and placed on a rocker platform for 16 h at 25 °C. The membranes were washed once for 15 min and then three times for 5 min each in TTBST. Immunoreactive bands were detected by enhanced chemiluminescence (utilizing ECL plus chemiluminescence detection reagents from Amersham) and subsequent exposure to X-ray film.

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