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Synthesis of nuclease-resistant siRNAs possessing universal overhangs

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ABSTRACT

RNA interference (RNAi) induced by small interfering RNA (siRNA) has emerged as a powerful technique for the silencing of gene expression at the post-transcriptional level. It has been shown that in the RNAi machinery, the 3′-overhang region of a guide strand (an antisense strand) of siRNA is recognized by the PAZ domain in the Argonaute protein, and the 2-nucleotide (nt) 3′-overhang is accommodated into a binding pocket composed of hydrophobic amino acids in the PAZ domain. Based on this background information, we designed and synthesized siRNAs possessing aromatic compounds at their 3′-overhang regions. It was found that the modified siRNAs possessing aromatic compounds are more potent than the siRNAs without the 3′-overhang regions. Further, the silencing activities of the modified siRNAs are almost equal to those of normal siRNAs with natural nucleosides at their 3′-overhang regions. We also found that the siRNAs possessing the aromatic compounds at their 3′-overhang region could be used to inhibit hepatitis C virus (HCV) replication. Moreover, the RNAs with aromatic groups at their 3′-ends were more resistant to nucleolytic degradation by snake venom phosphodiesterase (SVPD) (a 3′-exonuclease) than natural RNAs. The aromatic compounds described in this report do not have functional groups capable of forming hydrogen bonds with nucleobases. Therefore, we expect that they can serve as the universal overhang units that can improve the nuclease resistance of siRNAs.

1. Introduction

RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing that is triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes.[1] The process is initiated by the processive cleavage of dsRNA into 21- to 23-nucleotide (nt) duplexes by the enzyme Dicer. These duplexes, which contain a 2-nt overhang at the 3′-end of each strand, are termed as short interfering RNAs (siRNAs). The siRNAs associate with the RNA-induced silencing complex (RISC), which is then guided to catalyze the sequence-specific degradation of the target mRNA.[2–4]

siRNA has considerable potential as a new therapeutic drug for intractable diseases because siRNAs can be rationally designed and synthesized if the sequences of the disease-causing genes are known. Several groups have demonstrated the efficacy of siRNA-mediated inhibition of clinically relevant genes in vivo as well as in vitro.[4–6] Improving the nuclease resistance of siRNA is important for the therapeutic application of synthetic siRNAs.[4] Thus far, many types of siRNAs modified at the base, sugar, or phosphate moieties have been synthesized, and their nuclease-resistant properties and RNAi-inducing activities have been studied.[7–26]

Argonaute2, a key component of RISC, is responsible for mRNA cleavage in the RNAi pathway.[27,28] Argonaute2 is composed of PAZ, Mid, and PIWI domains. X-ray structural analysis and a nuclear magnetic resonance (NMR) study have revealed that the 2-nt 3′-overhang region of the guide strand (the antisense strand) of siRNA is recognized by the PAZ domain and is accommodated into a binding pocket composed of hydrophobic amino acids; this pocket is located in the domain.[29–32] The length of the 3′-overhang regions of siRNA influences the activities of the 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.[33]

Considering this background information, we substituted the natural nucleosides at 3′-overhang regions with the aromatic compounds, 1,3-bis(hydroxymethyl)benzene (**1**), 1,3-bis(hydroxymethyl)pyridine (**2**), and 1,2-bis(hydroxymethyl)benzene (**3**) (Fig. 1). We hypothesized that the introduction of lipophilic groups

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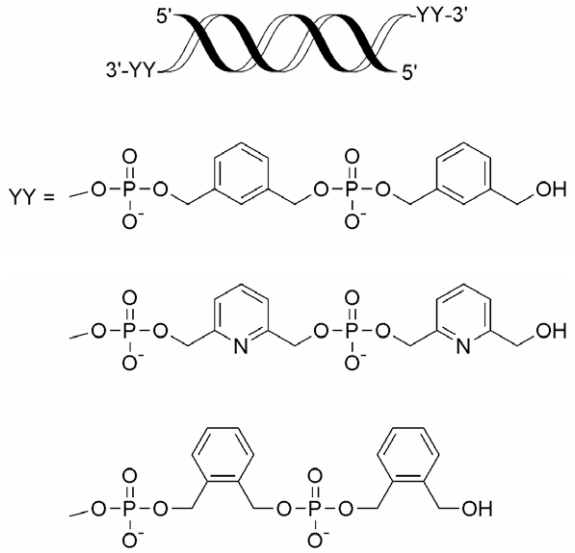


Figure 1. Structures of the siRNAs.

at the 3′-overhang portions of the siRNAs would improve affinities of the 3′-overhang portions of the siRNAs with the PAZ protein making the siRNAs more potent than normal siRNAs which possess natural nucleosides at their 3′-overhang portion. In addition, the modified RNAs would be more nuclease-resistant than the normal siRNAs.

In this paper, we report the synthesis and the silencing activities of the siRNAs with the aromatic groups **1**–**3** at their 3′-overhang regions. The nuclease-resistant properties of the siRNAs are also reported.

2. Results

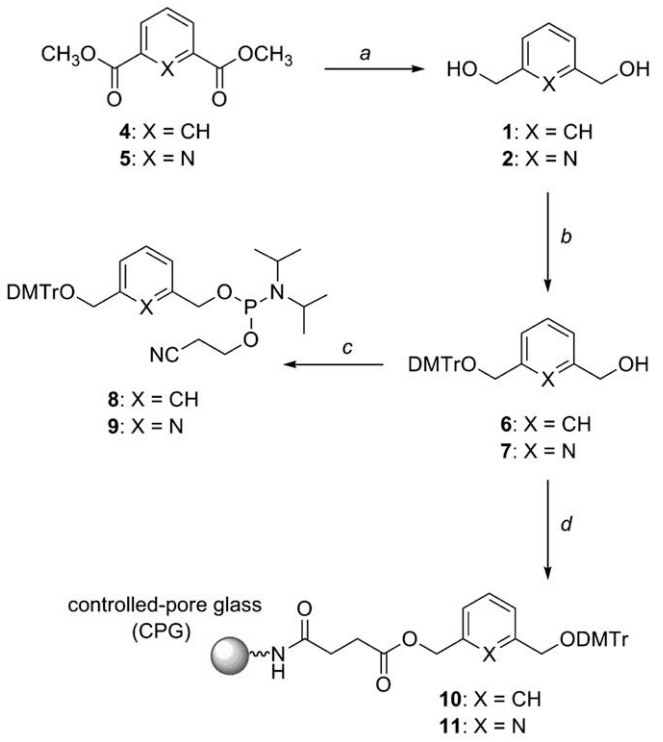
2.1. Synthesis

Modified siRNAs were synthesized by the phophoramidite method. In order to incorporate the aromatic compounds at the 3′-overhang regions of siRNAs, solid supports carrying **1**, **2**, or **3** and phosphoramidites of **1**, **2**, and **3** were synthesized according to routes shown in Schemes 1 and 2. Dimethyl isophtalate was treated with $LiBH_4$ to give 1,3-bis(hydroxymethyl)benzene (**1**) in 95% yield. One of the two hydroxyl groups of **1** was protected with a 4,4′-dimethoxytrityl (DMTr) group to afford a mono-DMTr derivative **6** in 51% yield. The mono-DMTr derivative **6** was phosphitylated by the standard procedure[34] to produce the corresponding phosphoramidite **8** in 94% yield. In a similar manner, the phosphoramidites **9** and **14** were synthesized from dimethyl 2,6-pyridinedicarboxylate and dimethyl phthalate with total yields of 13% and 64%, respectively.

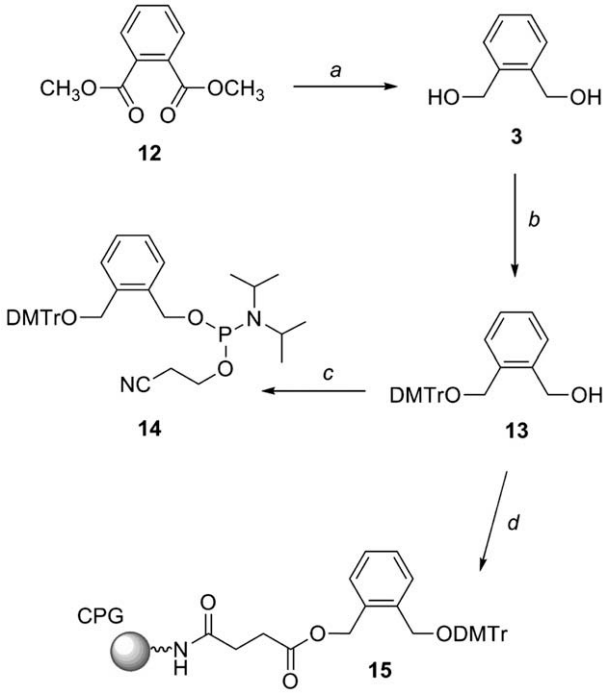
To enable attachment to the solid support, the mono-DMTr derivative **6** was succinated to yield the corresponding succinate, that was linked to controlled pore glass (CPG) to afford the solid support **10** possessing **6** (108 μmol/g). Similarly, the mono-DMTr derivatives **7** and **13** were succinated and then linked to the CPGs to afford the solid supports **11** and **15** possessing **7** (74 μmol/g) and **13** (86 μmol/g), respectively.

2.2. Oligoribonucleotide synthesis

All oligoribonucleotides (ONs) were synthesized with a DNA/RNA synthesizer. Fully protected ONs (1.0 μmol each) linked to solid supports were treated with concentrated NH_4OH:EtOH (3:1, v/v)



Scheme 1. Reagents and conditions: (a) $LiBH_4$, THF, rt, 95% for **1** and 28% for **2**; (b) DMTrCl, DMAP, pyridine, rt, 51% for **6** and 51% for **7**; (c) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *i*-Pr_2NEt, THF, rt, 94% for **8** and 93% for **9**; (d) (1) succinic anhydride, DMAP, pyridine, rt; (2) CPG, WSCI, DMF, rt, 108 μmol/g for **10** and 74 μmol/g for **11**.



Scheme 2. Reagents and conditions: (a) $LiBH_4$, THF, rt, 72%; (b) DMTrCl, DMAP, pyridine, rt, 90%; (c) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, *i*-Pr_2NEt, THF, rt, 98%; (d) (1) succinic anhydride, DMAP, pyridine, rt; (2) CPG, WSCI, DMF, rt, 86 μmol/g.

at room temperature for 12 h and then with 1.0 M tetra-*n*-butylammonium fluoride (TBAF)/THF at room temperature for 12 h.

The ONs that were released from the support after the treatment were purified using denaturing 20% polyacrylamide gel electrophoresis (PAGE) to afford deprotected ONs **36**–**77**. These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and the observed molecular weights were in agreement with their structures.

2.3. Thermal stabilities of siRNAs

The thermal stability of siRNAs was studied by thermal denaturation in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl (Table 2). The melting temperatures (T_ms) of siRNAs **18**, **21**, and **24** were 79.0, 77.1, and 78.2 °C, respectively. These results showed that the thermal stabilities of siRNA **21** which contained **1** and siRNA **24** which contained **2** were lower than that of siRNA **18** with a natural dinucleotide at its overhang portion. However, the T_m values of siRNAs **18**, **21**, and **24** were not considerably different from each other.

2.4. Dual-luciferase assay

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

Table 1
Sequences of oligonucleotides (ONs) used in this study

Number of siRNA	No. of ON	Sequence
siRNA**16**	ON**36**	5′-GGCCUUUCACUACUCCUAC-3′
	ON**37**	3′-CCGGAAAGUGAUGAGGAUG-5′
siRNA**17**	ON**38**	5′-GGCCUUUCACUACUCCUA*c*-3′
	ON**39**	3′-*t*CCGGAAAGUGAUGAGGAUG-5′
siRNA**18**	ON**40**	5′-GGCCUUUCACUACUCCUA*ct*-3′
	ON**41**	3′-*tt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**19**	ON**42**	5′-GGCCUUUCACUACUCCUA*ctt*-3′
	ON**43**	3′-*ttt*CCGGAAAGUGAUGAGGAUG-5′
siRNA**20**	ON**44**	5′-GGCCUUUCACUACUCCUAC**1**-3′
	ON**45**	3′-**1**CCGGAAAGUGAUGAGGAUG-5′
siRNA**21**	ON**46**	5′-GGCCUUUCACUACUCCUAC**11**-3′
	ON**47**	3′-**11**CCGGAAAGUGAUGAGGAUG-5′
siRNA**22**	ON**48**	5′-GGCCUUUCACUACUCCUAC**111**-3′
	ON**49**	3′-**111**CCGGAAAGUGAUGAGGAUG-5′
siRNA**23**	ON**50**	5′-GGCCUUUCACUACUCCUAC**2**-3′
	ON**51**	3′-**2**CCGGAAAGUGAUGAGGAUG-5′
siRNA**24**	ON**52**	5′-GGCCUUUCACUACUCCUAC**22**-3′
	ON**53**	3′-**22**CCGGAAAGUGAUGAGGAUG-5′
siRNA**25**	ON**54**	5′-GGCCUUUCACUACUCCUAC**222**-3′
	ON**55**	3′-**222**CCGGAAAGUGAUGAGGAUG-5′
siRNA**26**	ON**56**	5′-GGCCUUUCACUACUCCUAC**3**-3′
	ON**57**	3′-**3**CCGGAAAGUGAUGAGGAUG-5′
siRNA**27**	ON**58**	5′-GGCCUUUCACUACUCCUAC**33**-3′
	ON**59**	3′-**33**CCGGAAAGUGAUGAGGAUG-5′
siRNA**28**	ON**60**	5′-GGCCUUUCACUACUCCUAC**333**-3′
	ON**61**	3′-**333**CCGGAAAGUGAUGAGGAUG-5′
siRNA**29**	ON**62**	5′-AGAUCCAUACGUCCGUGAA*tt*-3′
	ON**63**	3′-*tg*UCUAGGUAUGCAGGCACUU-5′
siRNA**30**	ON**64**	5′-GCACCGGCAGGAGAUCAUA*tt*-3′
	ON**65**	3′-*gt*CGUGGCCGUCCUCUAGUAU-5′
–	ON**66**	**F**-5′-GGCCUUUCACUACUCCUA*ct*-3′
–	ON**67**	**F**-5′-GGCCUUUCACUACUCCUAC**11**-3′
siRNA**31**	ON**68**	5′-GUCUCGUAGACCGUGCAUCAUU-3′
	ON**69**	3′-UUCAGAGCAUCUGGCACGUAGU-5′
siRNA**32**	ON**70**	5′-GUCUCGUAGACCGUGCAUCA*tt*-3′
	ON**71**	3′-*tt*CAGAGCAUCUGGCACGUAGU-5′
siRNA**33**	ON**72**	5′-GUCUCGUAGACCGUGCAUCA**11**-3′
	ON**73**	3′-**11**CAGAGCAUCUGGCACGUAGU-5′
siRNA**34**	ON**74**	5′-GUCUCGUAGACCGUGCAUCA**12**-3′
	ON**75**	3′-**21**CAGAGCAUCUGGCACGUAGU-5′
siRNA**35**	ON**76**	5′-GUCUCAUAGGCCAUGCGUCA**11**-3′
	ON**77**	3′-**11**CAGAGUAUCCGGGACGCAGU-5′

The capital letters indicate ribonucleosides. The small italic letters represent 2′-deoxyribonucleosides. The underlined letters indicate the mismatch bases. **F** shows fluorescein.

Table 2
T_m values

siRNA	T_m (°C)
siRNA**18**	79.0
siRNA**21**	77.1
siRNA**24**	78.2

The results are shown in Figure 2. The silencing activities of the siRNAs possessing overhang moieties were markedly greater than those of siRNA **16**, which lacked an overhang portion. Surprisingly, the silencing activities of the siRNAs carrying aromatic compounds **1**, **2**, and **3** at their 3′-overhang regions were almost equal to those of siRNAs **17**, **18**, and **19**, which had natural thymidines at their 3′-overhang portions, at all concentrations. The number of aromatic derivatives at the overhang moieties seemed to influence the activities of the siRNAs. At all concentrations, the silencing activities of siRNAs with two aromatic compounds at their 3′-overhang regions tended to be greater than that of those carrying one or three aromatic compounds at their 3′-overhang portions. The silencing activities of the siRNAs carrying the 1,3-substituted compound **1**, the 1,2-substituted compound **2**, and the pyridine derivative **3** were not considerably different at each concentration.

Argonaute2/eIF2C2 (hAgo2) has been identified as a key protein with endonuclease activity associated with RISC in the RNAi pathway.[27,28] In order to examine whether the observed silencing activities could be attributed to RNAi, the activities of the modified siRNAs were studied after treating HeLa cells with eIF2C2-targeting siRNAs. We hypothesized that if the silencing activities of the modified siRNAs resulted from RNAi, the expression levels of luciferase proteins would recover if HeLa cells were treated with siRNAs that targeted eIF2C2. Two kinds of siRNAs targeting eIF2C2 were used in this study: one (siRNA **29**) that targeted open reading frame (ORF) positions 1168–1188 and another (siRNA **30**) that targeted ORF positions 1897–1917. HeLa cells were transfected with siRNA **29** or siRNA **30**. After incubation for 1 h, the cells were co-transfected with psiCHECK-2 vector and siRNAs modified with aromatic compounds. After incubation for 24 h, the activity of *Renilla* luciferase was measured. As shown in Figure 3, the signals of *Renilla* luciferase were recovered on treatment with siRNAs targeting eIF2C2. These results indicated that the silencing activities of modified siRNAs could be attributed to RNAi.

2.5. Nuclease-resistance

Improving the nuclease resistance of siRNA is important for the therapeutic application of synthetic siRNAs. It was expected that RNAs carrying the aromatic compounds would be more nuclease resistant than unmodified RNAs. First, the stability of the RNAs in PBS containing bovine serum was investigated. ONs **66** and **67** labeled at the 5′-ends with fluorescein were incubated in PBS containing 5% bovine serum. ON **67** contained the aromatic compound **1** at the 3′-overhang region. The reactions were analyzed with PAGE under denaturing conditions. Figure 4 shows the results. After 1 min of incubation, both the ONs were hydrolyzed completely. The results implied that ON **67**, which carried **1**, was hydrolyzed mainly by the endonuclease activity in the bovine serum.

Next, the susceptibility of the ONs to snake venom phosphodiesterase (SVPD), a 3′-exonuclease, was examined. Unmodified ON

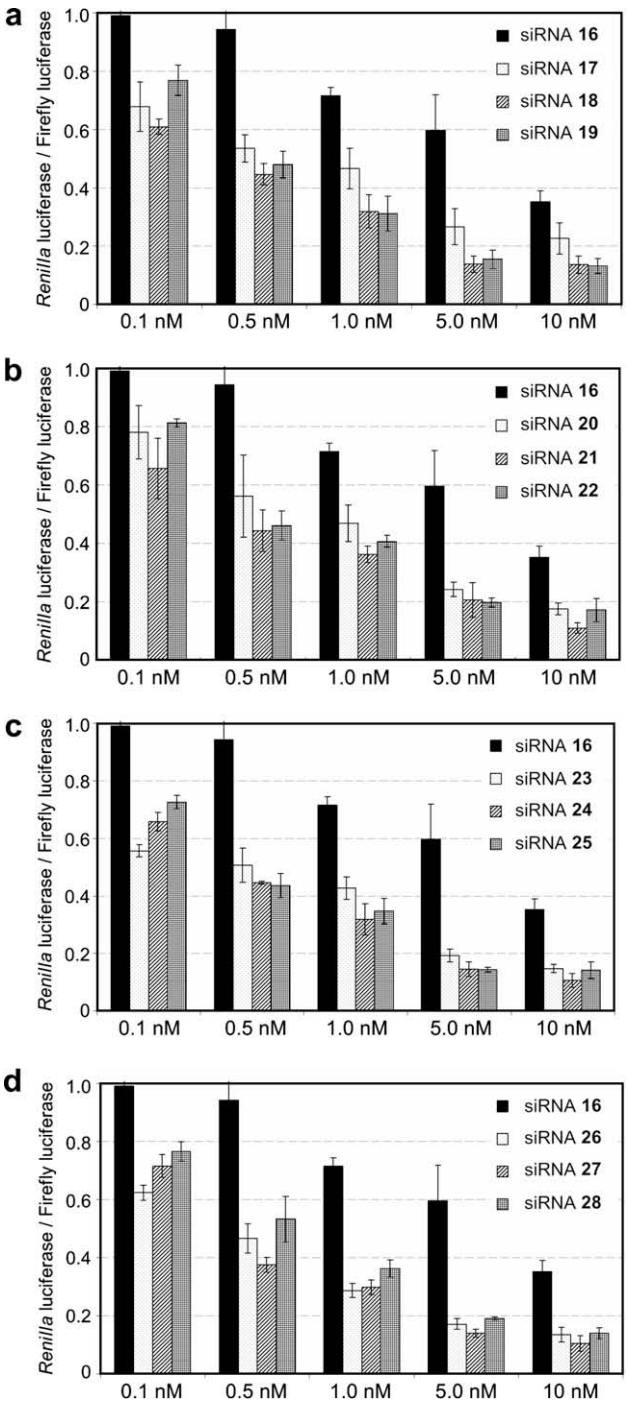


Figure 2. Dual-luciferase assay (1). The experimental conditions are as given under Section 4.

40, modified ON **46** carrying benzene derivative **1**, and modified ON **52** that contained pyridine derivative **2** were labeled with [γ-^{32}P]ATP and incubated with SVPD. The reactions were analyzed using PAGE under denaturing conditions. As shown in Figure 5, unmodified ON **40** was hydrolyzed randomly after 30 min of incubation, while modified ONs **46** and **52** were resistant to the enzyme. The half-lives ($t_{1/2}$s) of ONs **40**, **46**, and **52** were 7, 64, and 72 min, respectively. Therefore, it was apparent that ON **46** carrying benzene derivative **1** and ON **52** carrying pyridine derivative **2** were 9 and 10 times more resistant to SVPD than unmodified ON **40**.

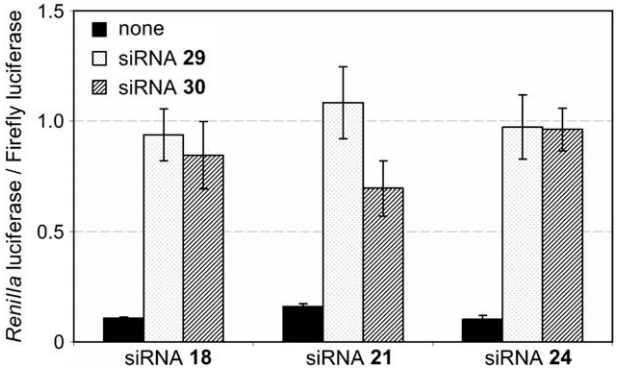


Figure 3. Dual-luciferase assay (2). HeLa cells were transfected with siRNA **29** (50 nM) or siRNA **30** (50 nM). After incubating for 1 h, the cells were co-transfected with a psiCHECK-2 vector and the modified siRNAs (10 nM). After incubating for 24 h, the activity of *Renilla* luciferase was measured. The experimental conditions are as given under Section 4.

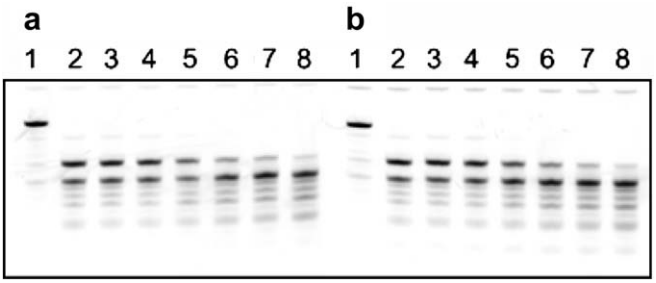


Figure 4. 20% PAGE of ONs incubated in PBS containing 5% bovine serum: (a) ON**66**; (b) ON**67**. ONs were incubated for 0 min (lane 1), 1 min (lane 2), 3 min (lane 3), 15 min (lane 4), 1 h (lane 5), 3 h (lane 6), 6 h (lane 7), and 12 h (lane 8). The experimental conditions are as given under Section 4.

2.6. Inhibition of HCV replication

It has been reported that hepatitis C virus (HCV) replication is efficiently suppressed by siRNA **31** that targets an internal ribosome entry site (IRES) region of HCV.[35] Accordingly, in order to examine the efficacy of the modified siRNAs, we compared the abilities of the modified siRNAs to suppress HCV replication with those of normal siRNAs. The sequences of the siRNAs used in this study are listed in Table 1. The siRNAs **32**, **33**, and **34** contained thymidines, benzene derivatives, and benzene and pyridine derivatives at their 3′-overhang regions, respectively. siRNA **35** contained 4 mismatched bases in its sequence.

Figure 6 shows the results. siRNAs **32**, **33**, and **34** dose-dependently inhibited HCV replication. They almost completely suppressed HCV replication at a concentration of 100 pM, while the replication ratio of the siRNA**35**, which contained the mismatched bases, was 75% at the same concentration. The siRNAs exerted no cytotoxic effect at 100 pM concentrations (Fig. 6c and d). Thus, it was found that modified siRNAs **33** and **34** suppress HCV replication in a sequence-specific manner. At each concentration, the replication ratios of HCV in cells treated with siRNAs **33** and **34**, which carried aromatic compounds at their 3′-overhang regions, were almost equal to the ratio in the cells treated with siRNAs **31** and **32** with natural uridines and thymidines at their 3′-overhang portions. These results indicated that the silencing abilities of the modified siRNAs are almost equal to those of the normal siRNA, which had natural nucleosides at their 3′-overhang regions.

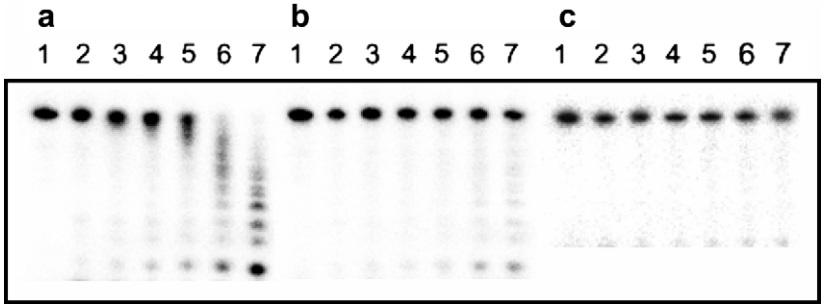


Figure 5. 20% PAGE of 5′-^{32}P-labeled ONs hydrolyzed by SVPD: (a) ON**40**, (b) ON**46**, (c) ON**52**. ONs were incubated with SVPD for 0 min (lane 1), 1 min (lane 2), 3 min (lane 3), 5 min (lane 4), 10 min (lane 5), 30 min (lane 6), and 60 min (lane 7). The experimental conditions are as described in Section 4.

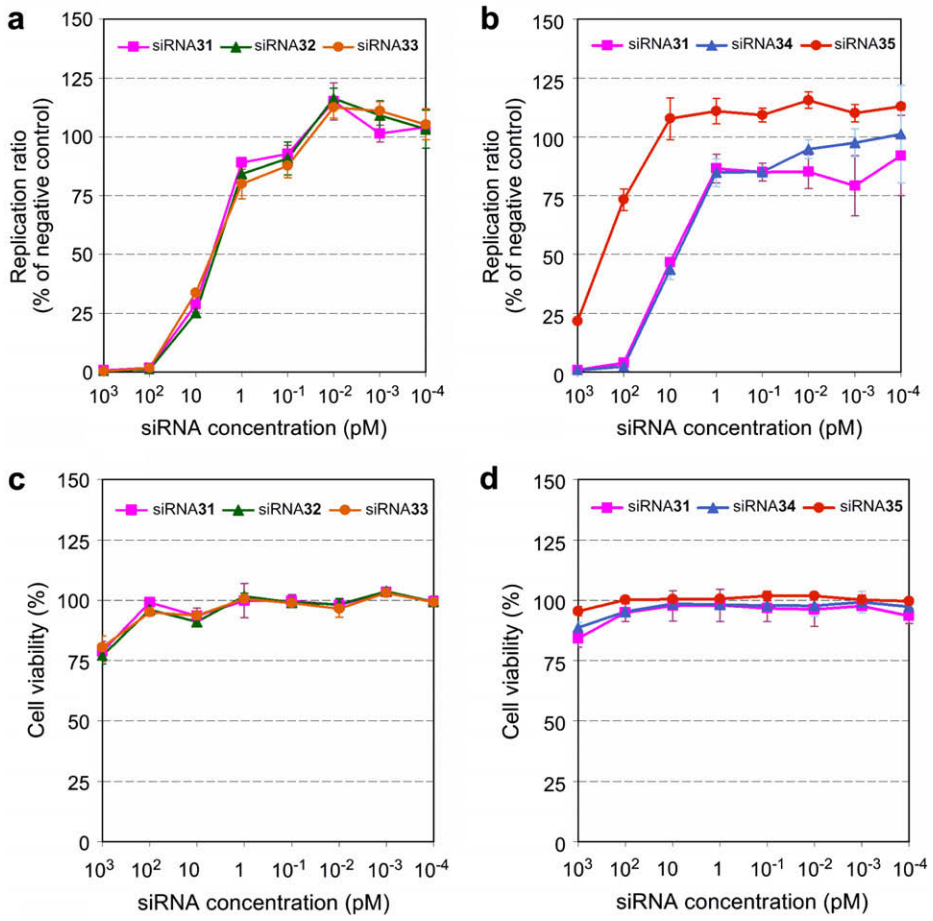


Figure 6. Effect of siRNAs on HCV replication. (a) and (b): Inhibition of HCV replication by siRNAs in R6FLR-N replicon cells. HCV replication was calculated by measuring the luminescence ratio with a Bright-Glo luciferase assay system. (c) and (d): Cell viability was determined by a WST-8 assay. Data are represented as mean ± SD (n = 3). The experimental conditions are as described in Section 4.

3. Discussion and conclusions

We designed and synthesized siRNAs possessing the aromatic compounds, 1,3-bis(hydroxymethyl)benzene, 1,3-bis(hydroxymethyl)pyridine, and 1,2-bis(hydroxymethyl)benzene at their 3′-overhang regions. RNAs containing these aromatic compounds at their 3′-ends were successfully synthesized by the phosphoramidite method by using a DNA/RNA synthesizer. It has been reported that the overhang nucleosides of RNA/RNA duplexes influence the thermal stabilities of the RNA/RNA duplexes.[36] Therefore, the thermal stability of the siRNAs was studied using thermal denaturation analysis. It was found that although the thermal stability of siRNA **21**, which contained 1,3-bis(hydroxymethyl)benzene, and siRNA **24**, which contained 1,3-bis(hydroxymethyl)pyridine, is slightly lower than that of siRNA **18**, which had a natural dinucleotide at the overhang portion, the T_m values of these three siRNA were not considerably different. Therefore, it was considered that the change in the thermal stability of the siRNAs, caused by the introduction of aromatic residues, influenced the silencing activities of the siRNAs to a very small extent.

The silencing activities of the siRNAs were examined by dual-luciferase assay using the psiCHECK-2 vector. It was revealed that the modified siRNAs were more potent than the siRNAs without the 3′-overhang regions. Moreover, the silencing activities of the

modified siRNAs were almost equal to those of the normal siRNAs. It has been reported that in the RNAi machinery, the 3′-overhang region of the guide strand (the antisense strand) of siRNA is recognized by a PAZ domain, and the 2-nt 3′-overhang is accommodated into a binding pocket composed of hydrophobic amino acids; this pocket is located in the domain.[29–32] In this report, we tested the siRNAs with the monocyclic compounds at their 3′-overhang regions. They showed silencing activities similar to those of unmodified siRNAs. Therefore, it is considered that recognition of the 3′-overhang portions of the siRNAs by the PAZ domain of human Argonaute is not so stringent. It has been reported that the 2-nt 3′-overhang was the most efficient in an experiment using 21-nt siRNA in *Drosophila* embryo lysate.[33] The silencing activity of the siRNAs with two aromatic compounds at their 3′-overhang regions also tended to be greater than that of carrying one or three aromatic compounds in this experiment.

It has been reported that pre-treatment of tissue culture cells with siRNAs can inhibit the replication of a large number of viruses.[37] Therefore, we examined the inhibition activity of the modified siRNAs using the cells harboring HCV replicons. It was found that the modified siRNAs suppressed HCV replication in a sequence-specific manner. The replication ratios of HCV in cells treated with siRNAs carrying aromatic compounds at their 3′-overhang regions were almost equal to those treated with siRNAs with natural nucleosides at their 3′-overhang portions. Therefore, siRNAs possessing aromatic compounds at their 3′-overhang region can be used to inhibit HCV replication.

An improvement in nuclease resistance of siRNA is important for the therapeutic application of synthetic siRNA.[4–6] We examined the susceptibility of the RNAs possessing aromatic compounds at their 3′-ends. SVPD was used as the model 3′-exonuclease, and the stability of the RNAs in bovine serum was also studied. The 3′-modified RNAs were hydrolyzed completely by the endonuclease activity in the bovine serum after 1 min of incubation. In contrast, the 3′-modification was effective for improving resistance to SVPD, a 3′-exonuclease.

In summary, we synthesized the siRNAs possessing the aromatic compounds at their 3′-overhang regions. It was revealed that the modified siRNAs were more potent than the siRNAs without 3′-overhang regions and that the modified siRNAs had silencing activity similar to those of the siRNAs possessing the natural nucleosides at their 3′-overhang portions. Moreover, the modified RNAs were more resistant to 3′-exonuclease than the natural RNAs. The aromatic compounds described in this report do not have functional groups capable of forming hydrogen bonds with nucleobases. Therefore, they can be used as universal overhang units in order to improve the nuclease resistance of siRNAs.

4. Experimental

4.1. General remarks

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

4.1.1. 1,3-Bis(hydroxymethyl)benzene (1)

A mixture of dimethyl isophtalate (2.00 g, 10.3 mmol) and $LiBH_4$ (1.12 g, 51.5 mmol) in THF (52 mL) was stirred at room temperature for 23 h. Aqueous $NaHCO_3$ (10 mL) was added to the mixture at 0 °C, and the whole was stirred at room temperature for 1 h. The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, EtOAc) to give **1** (1.36 g, 9.84 mmol) in 95% yield: ^{1}H NMR ($CDCl_3$) δ 7.39–7.26 (m, 4H), 4.71 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 141.2, 128.7, 126.2, 125.5, 65.1; LRMS (EI) *m/z* 138 (M^+); HRMS (EI) calcd for $C_8H_{10}O_2$ 138.0681, found 138.0677. Anal. Calcd for $C_8H_{10}O_2$: C, 69.54; H, 7.30. Found: C, 69.45; H, 7.23.

4.1.2. 1-(4,4′-Dimethoxytrityloxymethyl)-3-(hydroxymethyl) benzene (6)

A mixture of **1** (0.50 g, 3.62 mmol), DMTrCl (1.23 g, 3.62 mmol), and DMAP (22 mg, 0.18 mmol) in pyridine (18 mL) was stirred at room temperature for 17 h. The mixture was partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20% EtOAc in hexane) to give **6** (0.82 g, 1.86 mmol) in 51% yield: ^{1}H NMR ($CDCl_3$) δ 7.52–6.82 (m, 17H), 4.70 (s, 2H), 4.18 (s, 2H), 3.80 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 158.4, 145.0, 140.8, 139.7, 136.2, 130.1, 128.5, 128.2, 127.8, 126.7, 126.3, 125.7, 125.6, 113.1, 86.4, 65.4, 55.2; LRMS (EI) *m/z* 440 (M^+); HRMS (EI) calcd for $C_{29}H_{28}O_4$ 440.1988, found 440.1981. Anal. Calcd for $C_{29}H_{28}O_4 \cdot 1/5H_2O$: C, 78.27; H, 6.45. Found: C, 78.33; H, 6.59.

4.1.3. 1-[(2-Cyanoethyloxy)(*N*,*N*-diisopropylamino)phosphinyloxymethyl]-3-(4,4′-dimethoxytrityloxymethyl)benzene (8)

A mixture of **6** (0.35 g, 0.80 mmol), *N*,*N*-diisopropylethylamine (0.40 mL, 4.00 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (0.29 mL, 1.60 mmol) in THF (8 mL) was stirred at room temperature for 1 h. The mixture was partitioned between EtOAc and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (a neutralized SiO_2, 50% EtOAc in hexane) to give **8** (0.48 g, 0.75 mmol) in 94% yield: ^{31}P NMR ($CDCl_3$) δ 148.8; LRMS (FAB) *m/z* 641 (MH^+); HRMS (FAB) calcd for $C_{38}H_{46}N_2O_5P$: 641.3144, found: 6410.3129.

4.1.4. 2,6-Bis(hydroxymethyl)pyridine (2)

Dimethyl 2,6-pyridinedicarboxylate (2.00 g, 10.3 mmol) was treated as described in the preparation of **1** to give **2** (0.40 g, 2.88 mmol) in 28%: ^{1}H NMR ($CDCl_3$) δ 7.70 (t, 1H, *J* = 8.0), 7.19 (d, 2H, *J* = 8.0), 4.79 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 158.4, 137.4, 119.1, 64.3; LRMS (FAB) *m/z* 140 (MH^+); HRMS (FAB) calcd for $C_7H_{10}NO_2$ 140.0712, found 140.0705. Anal. Calcd for $C_7H_9NO_2$: C, 60.42; H, 6.52; N, 10.07. Found: C, 60.28; H, 6.50; N, 9.95.

4.1.5. 2-(4,4′-Dimethoxytrityloxymethyl)-6-(hydroxymethyl) pyridine (7)

Compound **2** (0.50 g, 3.59 mmol) was treated as described in the preparation of **6** to give **7** (0.81 g, 1.83 mmol) in 51%: ^{1}H NMR ($CDCl_3$) δ 7.76–6.82 (m, 16H), 4.69 (s, 2H), 4.34 (s, 2H), 3.79 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 158.5, 158.4, 157.6, 144.8, 137.3, 135.9, 130.0, 128.1, 127.9, 126.9, 119.4, 118.6, 113.2, 86.7, 66.6, 63.6, 55.20; LRMS (FAB) *m/z* 442 (MH^+); HRMS (FAB) calcd for $C_{28}H_{28}NO_4$: 442.2018, found: 442.2033.

4.1.6. 1-[(2-Cyanoethyloxy)(*N*,*N*-diisopropylamino)phosphinyloxymethyl]-3-(4,4′-dimethoxytrityloxymethyl)pyridine (9)

Compound **7** (0.20 g, 0.45 mmol) was phosphitylated as described in the preparation of **8** to give **9** (0.27 g, 0.42 mmol) in 93%: ^{31}P NMR ($CDCl_3$) δ 149.1; LRMS (FAB) *m/z* 642 (MH^+); HRMS (FAB) calcd for $C_{37}H_{45}N_3O_5P$: 642.3097, found: 642.3116.

4.1.7. 1,2-Bis(hydroxymethyl)benzene (3)

Dimethyl phthalate (2.00 g, 10.3 mmol) was treated as described in the preparation of **1** to give **3** (1.02 g, 7.38 mmol) in 72%: ^{1}H NMR ($CDCl_3$) δ 7.30 (s, 4H), 4.65 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 139.3, 129.6, 128.5, 63.9; LRMS (FAB) *m/z* 139 (MH^+); HRMS (FAB) calcd for $C_8H_{11}O_2$ 139.0765, found 139.0759. Anal. Calcd for $C_8H_{10}O_2$: C, 69.54; H, 7.30. Found: C, 69.75; H, 7.32.

4.1.8. 1-(4,4′-Dimethoxytrityloxymethyl)-2-(hydroxymethyl) benzene (13)

Compound **3** (0.50 g, 3.62 mmol) was treated as described in the preparation of **6** to give **13** (1.44 g, 3.27 mmol) in 90%: ^{1}H NMR ($CDCl_3$) δ 7.50–6.85 (m, 17H), 4.43 (d, 2H, *J* = 6.0), 4.18 (s, 2H), 3.79 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 158.6, 144.5, 140.5, 136.4, 135.7, 129.9, 129.8, 129.5, 128.6, 128.1, 127.9, 126.9, 113.4, 87.6, 65.2, 63.6, 55.2; LRMS (EI) *m/z* 440 (M^+); HRMS (EI) calcd for $C_{29}H_{28}O_4$ 440.1988, found 440.1991. Anal. Calcd for $C_{29}H_{28}O_4 \cdot 1/5H_2O$: C, 78.27; H, 6.45. Found: C, 78.24; H, 6.61.

4.1.9. 1-[(2-Cyanoethyloxy)(*N*,*N*-diisopropylamino)phosphinyloxymethyl]-2-(4,4′-dimethoxytrityloxymethyl)benzene (14)

Compound **13** (0.35 g, 0.80 mmol) was phosphitylated as described in the preparation of **8** to give **14** (0.50 g, 0.78 mmol) in 98%: ^{31}P NMR ($CDCl_3$) δ 148.4; LRMS (FAB) *m/z* 641 (MH^+); HRMS (FAB) calcd for $C_{38}H_{46}N_2O_5P$: 641.3144, found: 641.3127.

4.2. Solid support synthesis

A mixture of **1** (0.30 g, 0.68 mmol), succinic anhydride (0.20 g, 2.04 mmol), and DMAP (4 mg, 0.03 mmol) in pyridine (6.8 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 (0.50 g, 0.11 mmol) was added to a solution of the succinate and WSCI (0.11 g, 0.57 mmol) in DMF (10 mL), and the mixture was kept for 72 h at room temperature. After the resin was washed with pyridine, a capping solution (6 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 **1** to solid support was 108 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 **2** and **3** were obtained in 74 and 86 μmol/g loading amounts, respectively.

4.3. 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**44** (8), ON**45** (8), ON**46** (8), ON**47** (12), ON**48** (5), ON**49** (6), ON**50** (28), ON**51** (28), ON**52** (29), ON**53** (14), ON**54** (35), ON**55** (20), ON**56** (11), ON**57** (4), ON**58** (5), ON**59** (7), ON**60** (3), ON**61** (2), ON**66** (20), ON**67** (11), ON**72** (14), ON**73** (14), ON**74** (33), ON**75** (37), ON**76** (21), ON**77** (19). 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.[38]

4.4. MALDI-TOF/MS analysis of RNAs

Spectra were obtained with a time-of-flight mass spectrometer. ON**44**: calculated mass, 6094.6; observed mass, 6086.2. ON**45**: calculated mass, 6403.9; observed mass, 6397.7. ON**46**: calculated mass, 6294.8; observed mass, 6294.4. ON**47**: calculated mass, 6604.0; observed mass, 6605.6. ON**48**: calculated mass, 6494.9; observed mass, 6489.2. ON**49**: calculated mass, 6804.1; observed mass, 6797.1. ON**50**: calculated mass, 6095.6; observed mass, 6096.8. ON**51**: calculated mass, 6404.9; observed mass, 6405.0. ON**52**: calculated mass, 6296.6; observed mass, 6296.1. ON**53**: calculated mass, 6605.8; observed mass, 6603.5. ON**54**: calculated mass, 6497.9; observed mass, 6503.5. ON**55**: calculated mass, 6807.1; observed mass, 6807.2. ON**56**: calculated mass, 6094.6; observed mass, 6092.2. ON**57**: calculated mass, 6403.9; observed mass, 6399.1. ON**58**: calculated mass, 6294.8; observed mass, 6290.4. ON**59**: calculated mass, 6604.0; observed mass, 6603.7. ON**60**: calculated mass, 6494.9; observed mass, 6492.7. ON**61**: calculated mass, 6804.2; observed mass, 6806.8. ON**66**: calculated mass, 7070.4; observed mass, 7067.5. ON**67**: calculated mass, 6862.3; observed mass, 6856.9. ON**72**: calculated mass, 6739.9; observed mass, 6731.8. ON**73**: calculated mass, 6802.9; observed mass, 6794.6. ON**74**: calculated mass, 6740.9; observed mass, 6734.9. ON**75**: calculated mass, 6803.9; observed mass, 6797.9. ON**76**: calculated mass, 6739.9; observed mass, 6734.2. ON**77**: calculated mass, 6802.9; observed mass, 6794.7.

4.5. Thermal denaturation study

Each solution containing each siRNA (3 μM) in a buffer composed of 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) and 100 mM NaCl was heated at 95 °C for 3 min, then cooled gradually to an appropriate temperature, and used for the thermal denaturation studies. Thermal-induced transitions of each mixture were monitored at 260 nm with a spectrophotometer.

4.6. 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. Transfection without siRNA was used as a control. 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 a 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 mean ± SD.

4.7. Stability of ON in the PBS containing bovine serum

Each ON (600 pmol) labeled with fluorescein at 5′-end was incubated in PBS (300 μL) containing 5% bovine serum at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (15 μL). The mixtures were analyzed by electrophoresis on 20% polyacrylamide

gel containing 7 M urea. The labeled ON in the gel was visualized by a Typhoon system (Amersham Biosciences).

4.8. Partial hydrolysis of ONs with snake venom phosphodiesterase

Each ON (100 pmol) labeled with ^{32}P at the 5′-end was incubated with snake venom phosphodiesterase (3 ng) in a buffer containing 37.5 mM Tris-HCl (pH 7.0) and 7.5 mM $MgCl_2$ (total 40 μL) at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (10 μL). The mixtures were analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea. Densities of radioactivity of the gel were visualized by a Bio-imaging analyzer (Bas 2000, Fuji Co., Ltd).

4.9. Subgenomic HCV replicon cells

Subgenomic HCV replicon cells (R6FLR-N) were conditional expression system of the HCV-non-structure region and luciferase gene. These cells were cultured in DMEM-GlutaMAX High glucose (GIBCO) supplemented with 10% FBS, 1 unit penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), and 500 μg/ml G418 (GIBCO).

4.10. Transfection and evaluation of virus replication

Six kinds of siRNAs were used in this investigation. The siControl Non-Targeting siRNA (product No. D-001210-03-05; Dharmacon Inc.) was used as a negative control. The siRNA**31** was used as a positive control.[35] The subgenomic HCV replicon cells (R6FLR-N) were transfected with the siRNAs by reverse transfection. The cells were plated in 96-well plate (Falcon) at a density of 4×10^3 cells/well. Each siRNA (100 aM ~ 1 nM) was transfected to the cells using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (GIBCO-BRL). The cells were incubated for 72 h after being transfected with siRNAs. HCV replication was evaluated by luminescence in a Mithras LB940 (Berthold Technologies, Wildbad, Germany) using Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol.

4.11. Cell viability

In order to evaluate cytotoxic effects of the siRNAs, cell viabilities were measured by metabolic conversion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufactuer's protocol.

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