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Synthesis of modified siRNA bearing C-5 polyamine-substituted pyrimidine nucleoside in their 3′-overhang regions and its RNAi activity

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ABSTRACT

Short interfering RNA (siRNA) induces specific gene silencing by the RNA interference (RNAi) pathway. Nucleosides in the 3′-overhang regions of siRNAs were replaced with 5-bis(aminoethyl)aminoethylcarbamoylmethyl-2′-deoxyuridine or thymidine. siRNA bearing modified nucleoside was more active in silencing the gene expression of hepatocyte nuclear factor 4α (HNF4α) compared with siRNA bearing thymidine.

Targeted gene silencing through RNA interference (RNAi) opens up exciting possibilities in gene technology.[1] In nature, this ancient and evolutionarily conserved viral defense mechanism consists of short interfering RNA (siRNA), sometimes known as small interfering RNA or silencing RNA, which is a class of double-stranded RNA molecules enzymatically generated by the Dicer protein from longer RNAs of viral origin. These siRNAs interact with several proteins to form an RNA-induced silencing complex (RISC), which recognizes corresponding sequences in mRNA, causes degradation of the sequences, and interferes with expression of a specific gene. This silences gene expression without triggering non-specific RNA degradation and translation inhibition based on the interferon response. Thus, siRNA empowers researchers with a highly specific tool to ablate the gene of interest and has proven to be a robust agent for analysis of biological function in cell culture. siRNAs also have considerable potential as new therapeutic drugs for intractable diseases because they can be rationally designed and prepared on the basis of genes related to disease. Various siRNAs that are stable against nuclease have been designed for use as therapeutic candidates. siRNA with a modified nucleoside in its 3′-overhang regions is an example of such modified siRNAs.[2] The 2-nt 3′-overhang region is considered the most efficient in an experiment using 21-nt siRNA, and this 2-nt region of the guide strand of siRNA is recognized by the PAZ domain, which is found in Argonaute2, a key component of RISC.[3] In addition, most commercial available siRNA had thymidines in their 3′-overhang region.

Previously, we reported the synthesis and properties of an oligodeoxyribonucleotide (ODN) bearing polyamine-substituted pyrimidine nucleoside at the C-5 position.[4] Matsukura et al. also found that ODN with C5-modified pyrimidine bases, particularly with a polyamine moiety (trisamine), exhibits a higher antisense activity as well as reduced cytotoxicity in cells infected with HIV-1.[5] Recently, RNA bearing 5-propynyl pyrimidine nucleoside in the region complementary to target mRNA was found to be detrimental to RNAi activity due to the bulkiness.[6]

Hepatocyte nuclear factor 4α (HNF4α), a member of the nuclear hormone receptor family, is one of the most abundant nuclear orphan receptors expressed in the liver. This receptor is involved in early liver development.[7] It is also expressed in kidney, intestine, and pancreas and is required for expression of many tissue-specific traits in all of these organs. Recent reports have demonstrated that siRNA for HNF4α can decrease expression of HNF4α and the DNA binding activity to the target promoters in HepG2 cells.[8]

Here, we synthesized siRNAs having modified nucleosides in their 3′-overhang regions. In in vitro experiments, these modified siRNAs were found to be more effective against HNF4α suppression compared with siRNAs without modification. We also report the nuclease-resistant properties of these modified RNAs.

Synthesis: We synthesized three duplex RNAs for these experiments. All of these RNAs contained modified nucleosides or deoxyribonucleosides in their 3′-overhang regions (Table 1). For these

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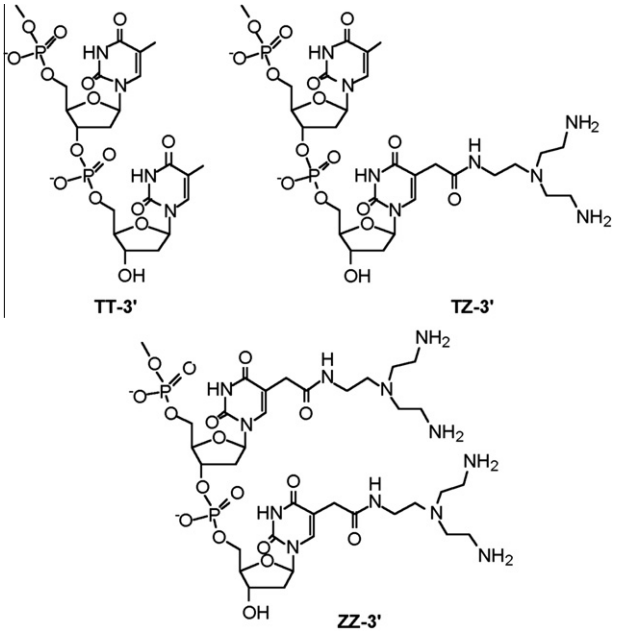
Table 1
Sequences and structures of modified siRNAs

dsRNA	ssRNA	Sequence
siRNA 1	**ORN** 1	5′-ggcagugcgugguggacaaTT-3′
	ORN 2	5′-uuguccaccacgcacugccTT-3′
siRNA 2	**ORN** 3	5′-ggcagugcgugguggacaaTZ-3′
	ORN 4	5′-uuguccaccacgcacugccTZ-3′
siRNA 3	**ORN** 5	5′-ggcagugcgugguggacaaZZ-3′
	ORN 6	5′-uuguccaccacgcacugccZZ-3′

T represent thymidine and Z represent 5-bis(aminoethyl)-aminoethyl-2′-deoxyuridine.

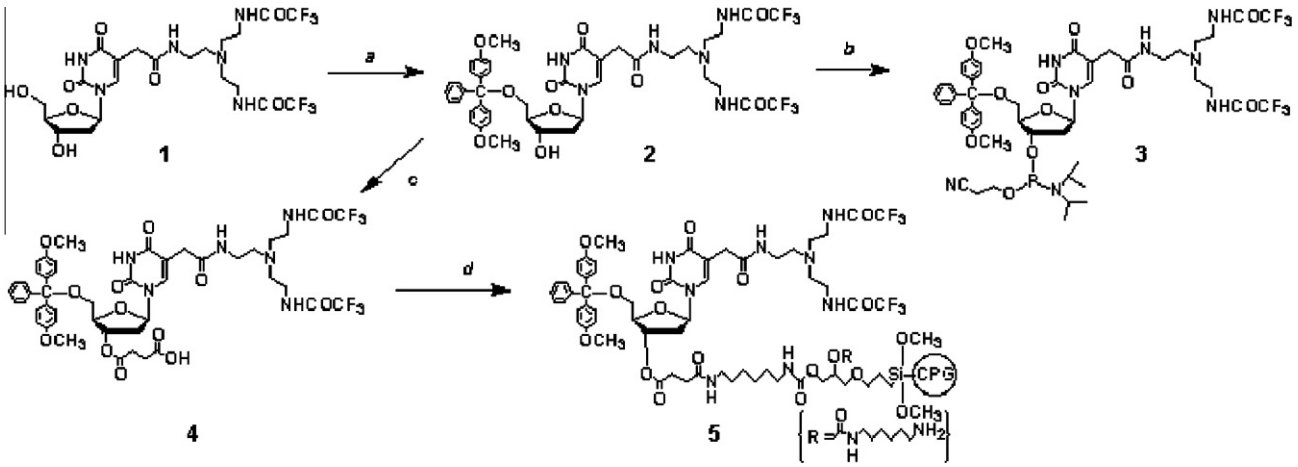


purposes, phosphoramidite **3** and modified solid support **5** were synthesized according to the routes shown in Scheme 1. First, modified nucleoside **1** was synthesized according to our previous report.[4] Nucleoside **1** was protected with a 4,4′-dimethoxytrityl (DMTr) group to obtain a mono-DMTr derivative **2** in 30% yield. Then, compound **2** was phosphitylated to obtain the corresponding phosphoramidite **3** by a standard procedure.[9] Phosphoramidite **3** was purified by chromatography on silica gel and eluted with ethyl acetate, dichloromethane, and triethylamine [4.5:4.5:1 (v/v)]. The yield of compound **3** was 60%.

Solid support was synthesized by a standard procedure.[10,11] 3′-Succinate **4** was synthesized from compound **2** and succinic anhydride. It was then immobilized to a long-chain alkylamine controlled-pore glass (LCAA-CPG) resin using 1-(3-dimethyaminopropyl)ethylcarbodiimide and DMAP in dry pyridine. The amount of loaded compound **2** was measured by quantifying the DMTr cation released by a solution of trichloroacetic acid.

RNA synthesis: The RNA sequence was designed on the basis of the study of HNF4α.[8] Six oligoribonucleotides (**ORN**s) were synthesized by the phosphoramidite method using a 392 DNA/RNA synthesizer (Applied Biosystems) on a 1-μmol scale (Table 1). Coupling time for this synthesis was 10 min. Phosphoramidites of thymidine (T) and CPG-T were used for **ORNs 1** and **2**, respectively, to incorporate T in the 3′-overhang region. Similarly, phosphoramidites of T and modified CPG **5** were used for **ORNs 3** and **4** and those of modified nucleoside **3** and modified CPG **5** were used for **ORNs 5** and **6**, respectively. After cleavage from support and deprotection, **ORN**s were purified by HPLC in two steps using an ODS-80Ts column (Tosoh, 0.46 × 25 cm), 100 mM TEAA, and CH_3CN to obtain **ORNs 1**, **2**, **3**, **4**, **5**, and **6** at optical densities of 0.1, 0.2, 2.5, 0.4, 36, and 4.8 (260 nm), respectively. **ORNs 1**, **2**, **3**, **4**, and **6** were repeatedly purified by HPLC to obtain the high purity sample. This purification steps caused the loss of **ORN** and their yields were low. Extinction coefficients of **ORN**s were calculated from those of mononucleotides according to the nearest-neighbor approximation method. Purified **ORN**s were verified by denaturing 12% polyacrylamide gel electrophoresis (data not shown). The result showed that RNAs had good purity. In the gel electrophoresis, the movement of modified RNAs bearing C-5 polyamine-substituted pyrimidine nucleosides (**ORNs 3**–**6**) was slower than that of others (**ORNs 1** and **2**) and the order of mobility was **ORNs 5** and **6** > **ORNs 3** and **4** > **ORNs 1** and **2**. This indicates that the movement of **ORN** bearing more cationic amino groups was slow because the net charge was low.

Nuclease-resistant property: We investigated the nuclease-resistant property of **ORNs 1**, **3**, and **5** using snake venom phosphodiesterase (Worthington Biochemical Corp., SVPD), a 3′-exonuclease. Each **ORN** (0.8 nmol) was incubated with SVPD (0.01 U) in a buffer containing 100 mM $MgCl_2$ and 200 mM Tris–HCl (pH 8.0) at 37 °C for 5 and 30 min, respectively. The degraded products were ana-



Scheme 1. Synthesis of modified nucleoside phosphoramidite and modified solid support. Reagents and conditions: (a) DMTr-Cl, pyridine, rt; (b) 2-cyanoethyldiisopropylchlorophosphoramidite, *i*-Pr_2NEt, pyridine, CH_2Cl_2, rt; (c) succinic anhydride, DMAP, pyridine, rt; (d) LCAA-CPG, DMAP, 1-(3-dimethylaminopropyl)ethylcarbodiimide, pyridine, rt.

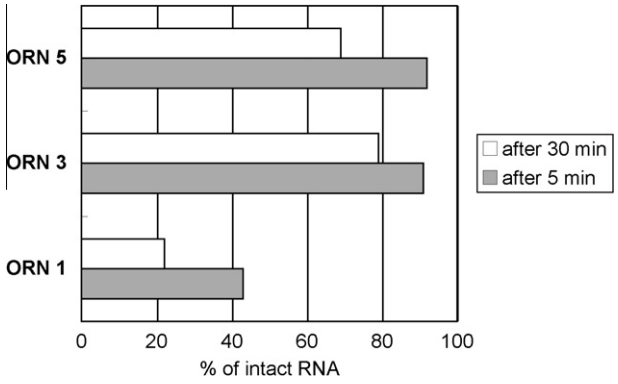


Figure 1. Nuclease-resistant activity of **ORNs 1**, **3**, and **5**.

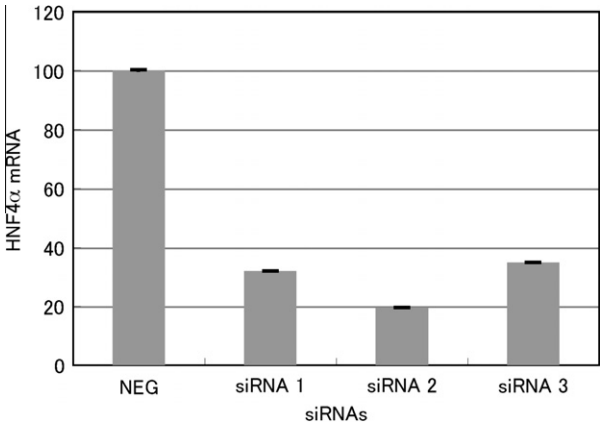


Figure 2. Real-time RT-PCR for measuring HNF4α mRNA. The cell was treated with siRNA (20 nM). NEG is nonsense control siRNA.

lyzed by RP-HPLC. The ratio of the remaining full-length RNA to the total RNA was calculated from the peak area on a chromatogram. Modified **ORNs 3** and **5** were more resistant compared with unmodified **ORN 1**, even after 30 min (Fig. 1). Because both **ORNs 3** and **5** with one and two modified nucleosides, respectively, in the 3′-overhang region showed nuclease resistance, it was concluded that only one modification in the 3′-overhang region is enough for resistance to exonuclease.

RNAi activity: The RNAi experiment for three siRNAs was conducted using human hepatoblastoma HepG2 cells, which express endogenous HNF4α at high level. Solutions of **siRNAs 1**, **2**, and **3** were prepared by annealing **ORN 1**/**ORN 2**, **ORN 3**/**ORN 4**, and **ORN 4**/**ORN 5**, respectively. These three siRNAs were evaluated for their activity against mRNA level of HNF4α by real-time RT-PCR.[12] The obtained results are shown in Figure 2 as the relative mRNA level to the level using control siRNA. **siRNA 2** was found to be more active against HNF4α compared with **siRNAs 1** and **3** (Fig. 2). In addition, the expression level of the HNF4α protein was confirmed by Western blotting (data not shown). These data showed that the expression level was decreased by treatment with **siRNA 2** compared with **siRNAs 1** and **3**.

These results suggest that only one modification in the 3′-overhang region is effective enough to increase siRNA activity. In the experiment conducted to determine nuclease-resistant property, the 3′-terminal modification was found to confer nuclease resistance to siRNA. Modification of two nucleosides in the 3′-overhang region resulted in same or slightly less RNAi activity. Modification of the second nucleoside in the 3′-overhang region might lead to poor recognition of mRNA.

In conclusion, we have prepared siRNAs bearing C-5 polyamine-substituted pyrimidine nucleosides in their 3′-overhang regions. RNAi activity of these modified siRNAs against the nuclear hormone receptor HNF4α was evaluated in HepG2 cells. Our results suggest that 3′-terminal modification improves RNAi activity. This could be due to the increased nuclease resistance caused by the modification.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.125.

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11. *Solid support synthesis*: Succinic anhydride (0.07253 g, 0.72 mmol), DMAP (30 mg, 0.25 mmol), and compound **2** (0.15 g, 0.17 mmol) were dissolved in dry pyridine (1 ml), and the obtained mixture was stirred at room temperature for 20 h. The mixture was then partitioned between CH_2Cl_2 and 5% aqueous solution of Na_2HPO_4, and the organic layer was coevaporated three times with toluene to remove pyridine. The desired corresponding succinate **4** was purified by column chromatography on silica gel, eluted with 10% MeOH in CH_2Cl_2, and finally precipitated in hexane (70 ml). The LCAA-CPG resin (3-Prime, 0.5 g) was then added to a solution of DMAP (6 mg, 0.05 mmol), 1-(3-dimethylaminopropyl)ethylcarbodiimide (191 mg, 1 mmol), and succinate **4** (20 mg, 0.04 mmol) in dry pyridine (5 ml). The mixture was shaken slowly for 4 h at room temperature after adding triethylamine (40 μl). After filtration, the resin was washed with dry pyridine and then with CH_2Cl_2 and left to dry. Finally, we obtained the modified solid support **5** on which the amount of loaded compound **2** was 23.68 μmol/g. The amount was measured by quantifying the DMTr cation released by a solution of 3% trichloroacetic acid in CH_2Cl_2.
12. *RNAi activity*: HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS at 37 °C. Each siRNA was then transfected into the cells with transfection reagent (Hily Max, DOJINDO). After incubation at 37 °C for 24 h, medium was changed to DMEM containing 10% FBS. After additional 24 h incubation, total RNA was extracted from the cells using RNAiso Plus (TaKaRa). The RNA was reverse-transcribed using ReverTraAce qPCR RT kit (TOYOBO). Real-time PCR was performed on a MyiQ Real-time PCR detection system (Bio-Rad). Purified cDNAs encoding HNF4α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified using THUNDERBIRD SYBR qPCR Mix (TOYOBO) with specific primers (HNF4α: 5′-CAGGCTCAAG AAATGCTTCC-3′ and 5′-GGCTGCTGTCCTCATAGCTT-3′; GAPDH: 5′-CAATGACC CCTTCATTGACC-3′ and 5′-GACAAGCTTCCCGTTCTCAG-3′). PCR was performed at 95 °C for 1 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each sample, the mean threshold cycle (Ct) from two replicate PCR using RNA isolated from independent cells was taken. Expression levels of HNF4α mRNA were normalized to GAPDH by the ΔΔCt method.