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Gene silencing by 2′-*O*-methyldithiomethyl-modified siRNA, a prodrug-type siRNA responsive to reducing environment

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ABSTRACT

RNAs bearing various 2′-modifications have been synthesized in an effort to improve nuclease resistance. However, the gene silencing activity of small interfering RNAs (siRNAs) has been decreased or sometimes completely suppressed by the chemical modifications. We previously developed a post-synthetic approach for the synthesis of 2′-*O*-methyldithiomethyl-modified RNA, which can be converted into unmodified RNA under reducing conditions, and named it Reducing-Environment-Dependent Uncatalyzed Chemical Transforming RNA (REDUCT RNA). Here, the gene silencing activity of REDUCT siRNA bearing 2′-*O*-methyldithiomethyl groups was evaluated. REDUCT siRNA showed more effective gene silencing than unmodified siRNA regardless of the modification site. This result suggests that REDUCT siRNA is converted into unmodified siRNA inside cells as a prodrug-type siRNA.

Since the discovery of RNA interference (RNAi),[1] small interfering RNAs (siRNAs) have emerged as a powerful molecular biological tool for sequence-specific gene silencing in mammalian cell lines.[2] siRNAs consist of 21–23 base pairs (bp) double-stranded RNA (dsRNA) with 2-nucleotide 3′-overhangs, and are incorporated into an RNA-induced silencing complex (RISC). In this process, helicase activity associated with RISC separates the two strands of the duplex,[3,4] releasing the sense (passenger) strand and permitting the binding of the antisense (guide) strand to complementary RNA to cause degradation of the target RNA.

RNAi-based therapy presents an attractive opportunity to engage targets not accessible to typical small molecules. However, RNAi needs strategies to overcome problems that hinder practical use. The fact that unmodified RNAs are rapidly catabolized by nucleases underscores the need for a high dose of native siRNA to reach cells. To circumvent this hurdle, numerous chemical modifications of RNA have been designed, such as 2′-*O*-Me, 2′-F, 2′,4′-BNA/LNA and so on.[5–9] A number of modifications have contributed to increasing the nuclease resistance of oligonucleotides, but at the same time, those modifications have decreased the gene silencing activities of siRNAs.[6–9] Indeed, fully 2′-*O*-methyl-modified siRNAs were virtually inactive.[10] Furthermore, modifications in the 5′-end and/or the seed region (positions 2–8 from 5′-end) of the antisense strand often decreased RNAi activity.[7,11] To overcome these drawbacks, we designed and synthesized a novel prodrug-type RNA, 2′-*O*-methyldithiomethyl (MDTM)-RNA,[12] which could be converted into unmodified RNA in the presence of 10 mM glutathione (GSH), pH 7.0, mimicking an intracellular reducing environment, and named it Reducing-Environment-Dependent Uncatalyzed Chemical Transforming RNA (REDUCT RNA) (Scheme 1).[12] This prodrug-type RNA possesses improved nuclease resistance as well.[12] In this study, we evaluated the gene silencing activity of siRNAs containing 2′-*O*-MDTM-uridine in mammalian cells.

Phosphoramidite units are required for the solid-phase synthesis of oligonucleotides. However, phosphoramidite units bearing alkyldithiomethyl groups are thought to be unstable, because the trivalent phosphorus atom on the phosphoramidites is capable of allowing an intramolecular attack on the disulfide bond.[13] Thus, we developed a novel post-synthetic approach to obtain 2′-*O*-MDTM-modified RNAs and prepared phosphoramidite unit **5** from uridine, as reported previously.[12]

By using **5** and commercially available phosphoramidite units protected with 2′-*O*-*tert*-butyldimethylsilyl (TBS) groups, oligoribonucleotides bearing one to four 2′-*O*-2,4,6-trimethoxybenzylthiomethyl (TMBTM)-uridines, as shown in Table 1, were synthesized on an automated DNA/RNA synthesizer in the trityl-off mode (Scheme 2). The synthesized oligonucleotides were deprotected and cleaved from the support by treatment with 28% aqueous ammonia/ethanol (3:1, v/v). After the ammonia treatment, the 2′-*O*-TBS groups were removed from the oligonucleotides with *N*-methyl-2-pyrrolidone/Et_3N/Et_3N–3HF. After HPLC

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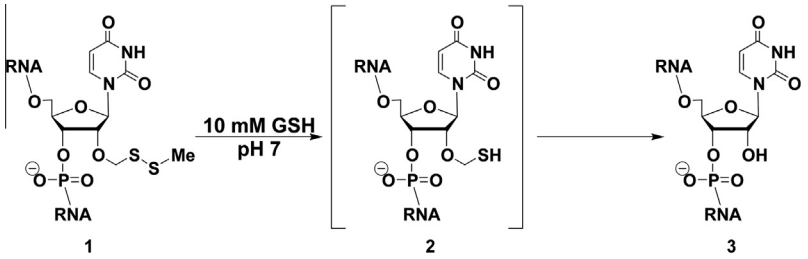


Scheme 1. Conversion of 2′-*O*-MDTM-RNA (**1**, REDUCT RNA) into unmodified RNA **3** in a reducing environment.

Table 1
Sequences of RNAs containing 2′-*O*-TMBTM-uridines and results of MALDI-TOF MS analysis

No.	Sequence (5′–3′)	MALDI-TOF MS [M−H]⁻	
		Calcd	Found
6a	CUU ACG CXG AGU ACU UCG ATT[a]	6832.3	6833.3
7a	CUU ACG CXG AGX ACU UCG ATT[a]	7144.7	7144.2
8a	CXU ACG CXG AGX ACU XCG ATT[a]	7511.1	7510.1
9a	UCG AAG UAC XCA GCG UAA GTT[a]	6918.4	6917.4
10a	UCG AAG XAC XCA GCG UAA GTT[a]	7144.7	7144.2
11a	XCG AAG XAC XCA GCG XAA GTT[a]	7597.2	7598.6

[a] X: 2′-*O*-TMBTM-uridine.

purification, 2′-*O*-TMBTM-RNAs **6a**–**11a** were obtained and characterized using MALDI-TOF mass spectrometry (Table 1).

To convert 2′-*O*-TMBTM-RNAs **6a**–**11a** into 2′-*O*-MDTM-RNAs **6b**–**11b** using the post-synthetic method, dimethyl(methylthio)-sulfonium tetrafluoroborate (DMTSF) was used (Scheme 2). Figure 1 shows the HPLC profiles of **6a** and **6b**. Other RNAs bearing 2′-*O*-TMBTM (**7a**–**11a**) were similarly converted into 2′-*O*-MDTM-RNAs **7b**–**11b** by treatment with DMTSF. 2′-*O*-MDTM-RNAs **6b**–**11b** were characterized using MALDI-TOF mass spectrometry (Table 2).

Substantially decreasing the thermal stability of siRNA may render siRNA ineffective. To evaluate the thermal stability of synthesized siRNAs, UV melting experiments were conducted. The results are summarized in Table 3. The 2′-*O*-MDTM modifications hardly affected the duplex stability of the siRNAs regardless of the modification site (Table 3). Next, we evaluated the conversion of double-stranded 2′-*O*-MDTM siRNAs into non-modified siRNAs under reductive conditions. We have already showed that single-stranded 2′-*O*-MDTM oligonucleotides are effectively converted into non-modified oligonucleotides in the presence of 10 mM GSH.[12] Thus, we investigated the conversion of double-stranded 2′-*O*-MDTM siRNAs in a similar manner. A typical example for the results is shown in Figure 2. siRNA 13, which contains four 2′-*O*-MDTM-modified uridines in the antisense strand, was effectively converted into non-modified siRNA 1. The result suggests that double-stranded 2′-*O*-MDTM siRNAs can be converted into double-stranded non-modified siRNAs in cells.

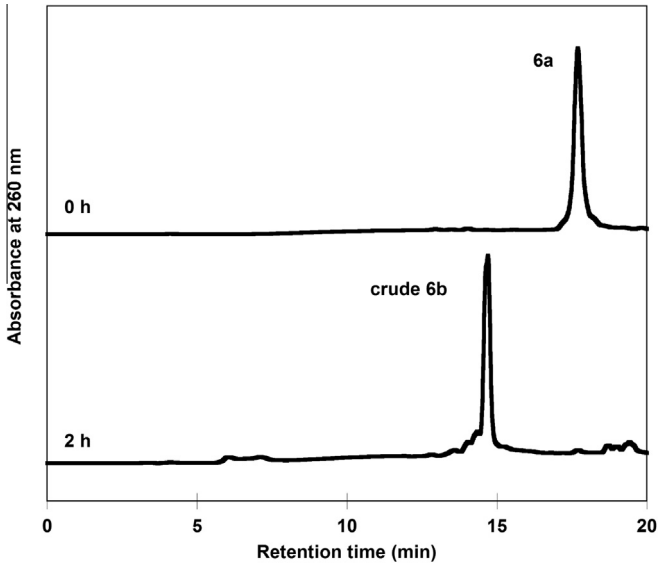
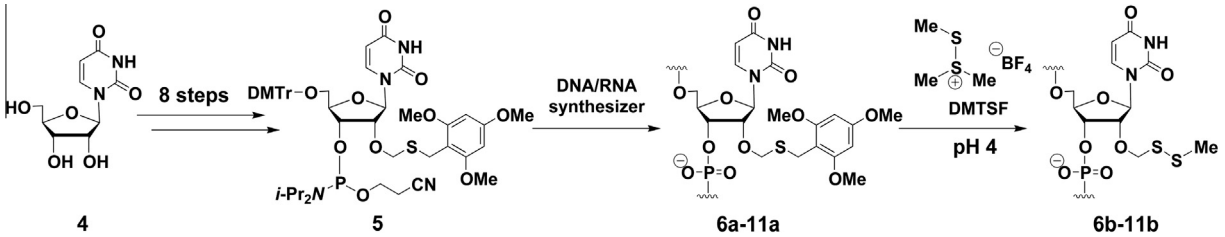


Figure 1. HPLC profiles of the conversion of **6a** into **6b** by treatment with DMTSF.

Table 2
Sequences of RNAs containing 2′-*O*-MDTM-uridines and results of MALDI-TOF MS analysis

No.	Sequence (5′–3′)	MALDI-TOF MS [M−H]⁻	
		Calcd	Found
6b	CUU ACG CUG AGU ACU UCG ATT[a]	6698.2	6698.4
7b	CUU ACG CUG AGU ACU UCG ATT[a]	6790.4	6790.3
8b	CUU ACG CUG AGU ACU UCG ATT[a]	6974.8	6974.9
9b	UCG AAG UAC UCA GCG UAA GTT[a]	6784.3	6784.4
10b	UCG AAG UAC UCA GCG UAA GTT[a]	6876.5	6876.6
11b	UCG AAG UAC UCA GCG UAA GTT[a]	7060.8	7060.6

[a] U: 2′-*O*-MDTM-uridine.



Scheme 2. Synthesis of 2′-*O*-TMBTM-RNAs **6a**–**11a** and their conversion into 2′-*O*-MDTM-RNAs **6b**–**11b**.