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RNA interference (RNAi) induced by siRNAs modified with 4’-thioribonucleosides in cultured mammalian cells

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Abstract: Short interfering RNAs (siRNAs) variously modified with 4’-thioribonucleosides against the *Photinus luciferase* gene were tested for their induction of the RNA interference (RNAi) activity in cultured NIH/3T3 cells. Results indicated that modifications at the sense-strand were well tolerated for RNAi activity except for full modification with 4’-thioribonucleosides. However, the activity of siRNAs modified at the antisense-strand was dependent on the position and the number of modifications with 4’-thioribonucleosides. Since modifications of siRNAs with 4’-thioribonucleosides were well tolerated in RNAi activity compared with that of 2’-*O*-methyl nucleosides, 4’-thioribonucleosides might be potentially useful in the development of novel and effective chemically modified siRNAs.

Key words: RNA interference (RNAi); Short interfering RNA (siRNA); Chemical modification; 4’-thioribonucleoside; 4’-thioRNA

Abbreviations: siRNA, short interfering RNA; RNAi, RNA interference; RISC, RNA-induced silencing complex

1. Introduction

RNA interference (RNAi) is a highly conserved biological response to double-stranded RNA that mediates post-transcriptional gene silencing [1]. After the discovery of RNAi by short interfering RNAs (siRNAs) [2], which are approximately 21 nucleotides in length with 3’ nucleotides overhang, much effort has been dedicated to the application of siRNAs not only as biological tools but also as therapeutic agents. However, since RNA is a transient molecule in biological fluids, the development of a chemically modified siRNA that conferred nuclease resistance would be a tremendously valuable tool. Thus far, several groups have reported RNAi activity of siRNAs with various chemical modifications (reviewed in [3], [4–9]). These approaches are mainly divided into two classes, i.e., modifications of the phosphodiester-backbone [4–6,8] and the ribose moiety [5–9]. In the first case, modification with phosphorothioates [5,6,8] and boranophosphates [4] has been examined. Hall et al. [4] found that boranophosphate siRNAs are consistently more effective than phosphorothioate siRNAs . Furthermore, boranophosphate siRNAs are frequently more active than natural siRNA if the center of the antisense-strand is not modified. As for the ribose moiety, siRNAs modified with 2’-deoxy [6], 2’-*O*-methyl [5,6,8,9] and 2’-fluoro [5–7] groups also have been investigated for RNAi activity. Several groups [6, 7] have reported that 2’-fluoro modifications are well tolerated for RNAi activity and enhance serum stability. Amarzguioui et al. [8] reported that certain siRNA species with 2’-*O*-methyl modifications showed long-term RNAi activity. In addition, Czauderna et al. [9] found that the 2’-*O*-methyl modifications at specific positions improve the stability of siRNA in serum and are tolerated without any significant loss of RNAi activity. However, thus far no overwhelming information f or the best choice of modification of siRNA has come forth.

On the other hand, the synthesis and properties of oligonucleotides containing 4’-thioribonucleosides (4’-thioRNAs) (Fig. 1) have been reported by our lab [10] and others [11,12]. The 4’-thioRNA formed a thermally stable duplex with the complementary RNA and 4’-thioRNA, and showed high nuclease resistance, despite of possessing the 2’-OH groups. In addition,

structural analysis by CD spectra indicated that the 4'-thioRNA:4'-thioRNA duplex and 4'-thioRNA:RNA duplex adopt an A-form conformation as does the natural RNA duplex. These results prompted us to investigate whether modifications of siRNA with 4'-thioribonucleosides influenced RNAi inducing activity. We report herein the first study of the structure-activity relationship of siRNAs modified with 4'-thioribonucleosides for RNAi activity.

2. Materials and methods

2.1. Preparation of siRNA modified with 4'-thioribonucleosides

Oligonucleotides containing 4'-thioribonucleosides [13] were synthesized on a DNA synthesizer (Applied Biosystems Model 392), as we previously reported [10]. For preparation of duplexes, sense- and antisense-strand oligonucleotides (20 μM each) were mixed together in an annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) and incubated for 1 min at 90 ºC then overnight at room temperature. All siRNA duplexes were synthesized with 2 nt deoxythymidine 3'-overhangs and directed against *photinus* luciferase, as described by Elbashir et al. [2]. Scramble siRNA duplex (B-bridge) was used as a negative control.

2.2. Cell culture, transfection, and dual luciferase assay

NIH/3T3 cells were grown at 37 ºC in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four hours before transfection at 70-90% confluency, cells were diluted with fresh medium (2 x 10^5 cells mL^{-1}) and transferred to 24-well plates (500 μL per well). Co-transfection of reporter plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies), as described by the manufacturer. Before co-transfection, the culture medium was replaced with 0.5 mL of fresh medium without antibiotics, and to each well 1.0 μg pGL2-Control (Promega), 0.1 μg pRL-TK (Promega), and siRNA duplex formulated into liposomes were applied; the final volume was 600 μL per well. After a 6 hour

incubation at 37 ºC, the culture medium was replaced with 0.5 mL of fresh medium supplemented with 10% FBS and antibiotics, and further incubated at 37 ºC. Twenty-four hours after transfection, the cell lysate was prepared and luciferase expression was subsequently monitored with the Dual-Luciferase reporter assay system (Promega). The IC_{50} value of each siRNA was estimated from a seven-point dose-response analysis.

3. Results

3.1. RNAi inducing activity of siRNA modified with 4’-thioribonucleosides

We prepared modified siRNA directed against *photinus* luciferase as described by Elbashir et al. [2]. We carried out the siRNA-luciferase reporter assay and investigated whether modifications of siRNA with 4’-thioribonucleosides influenced RNAi inducing activity in NIH/3T3 cells. The sequences, RNAi activity at 25 nM, and IC_{50} values of modified siRNA are shown in Table 1. The siRNAs (RNA2–RNA4) partially modified with 4’-thioribonucleosides at the sense-strand (upper strand) almost equaled natural siRNA (RNA1) in RNAi activity. RNAi activity was still observed even when all the residues of the sense-strand were modified (RNA5). On the other hand, the activity was drastically affected by the modification at the antisense-strand (lower strand). The RNAi activity was reduced to some degree when uridine or cytidine residues were substituted for 4’-thiouridine or 4’-thiocytidine (RNA6 and RNA7). From the comparison of IC_{50} values, their activities were 15–20 fold less than that of RNA1, however they were still effective for gene suppression. Further modifications with 4’-thioribonucleosides resulted in a significant loss of RNAi activity (RNA8 and RNA9).

We next tested RNAi using siRNAs consecutively modified with 4’-thioribonucleosides on their 5’-end, 3’-end and both ends, and central position (RNA10–RNA17). This mode of modification, especially with RNA13 and RNA17, was carried out according to the strategy for antisense molecules, namely a gapmer [14,15]. All of the modified siRNAs on their sense-strand (RNA10–RNA13) were almost equal to the natural siRNA (RNA1) in RNAi activity.

On the other hand, the RNAi activity was reduced somewhat compared with that of RNA1 when the 5'- or 3'-end of the antisense strand was modified (RNA14 and RNA15). The RNAi activity of RNA16, modified on the central position of the antisense strand, was fairly lower than RNA14 and RNA15, although the number of modification was nearly equal. Furthermore, modification of the both ends (RNA17) resulted in loss of RNAi activity. These results were almost compatible with the case of RNA2–RNA9.

In order to evaluate the utility of the chemical modification of siRNA with 4'-thioribonucleosides, time-course experiments of RNAi activity were examined. As sh own in Fig. 2, the RNAi activity of the natural siRNA (RNA1) was still the most effective after 3 days in comparison with those of RNA4 and RNA13. However, after 5 days, the modified siRNAs showed higher RNAi activity than natural RNA1. We next compared the RNAi activity of siRNAs modified with 2'-*O*-methyl nucleosides, i.e., RNA18–RNA21 vs. RNA10, RNA11, RNA14 and RNA15. Modification with 2'-*O*-methyl nucleosides is recognized as a promising candidate for chemically modified siRNA [8,9]. The siRNAs modified with 2'-*O*-methyl nucleosides on the sense-strand (RNA18 and RNA19), like RNA10 and RNA11, showed potent RNAi activity. On the other hand, the RNAi activity was reduced significantly when the antisense strand was modified (RNA20 and RNA21) relative to RNA1, and was much lower than the activity of RNA14 and RNA15. These results indicate that modification with 4'-thioribonucleosides were well tolerated in RNAi activity compared to that with 2'-*O*-methyl nucleosides, at least, in a certain type of modification.

3.2. Effects of additional mechanism-based modifications of siRNA molecules

The RNAi machinery is complex. Although still unclear, RNAi induced by siRNA is thought to require: 1) phosphorylation of the 5'-end(s) of siRNA by unknown kinase(s), 2) incorporation of the phosphorylated siRNA into RISC (RNA-induced silencing complex) via multiple steps starting with unwinding by helicase, and 3) sequence selective cleavage of target RNA by endonuclease activity of the RISC (reviewed in [16]). As shown in the previous section, modification of siRNA with

4’-thioribonucleosides generally resulted in a decrease in RNAi activity proportional to the number of modifications, especially on the antisense-strand. Thus, we next investigated why modification with 4’-thioribonucleosides influenced RNAi activity through additional mechanism-based modifications of siRNA.

The first possibility suggested that the efficiency of the phosphorylation of the modified siRNA might affect its RNAi activity. Since the gene suppression is abolished when the 5’-end of the antisense-strand is blocked, and the 5’-end of the sense strand is not necessary to be phosphorylated [17,18], we compared the RNAi activity of the siRNAs phosphorylated at the 5’-end of the antisense strand (RNA23 and RNA25) with that of unphosphorylated siRNAs (RNA7 and RNA14). However, the RNAi activity of RNA23 and RNA25 was not restored relative to RNA7 and RNA14.

To further test the effect of the chemical modification with 4’-thioribonucleosides, we then concentrated on the thermal stability of siRNAs. Recently, Schwarz et al. reported that siRNA is an asymmetric molecule for RISC formation and the strand with relatively lower thermal stability in the 5’-end is preferentially incorporated into RISC [19,20]. This was also confirmed by Hohjoh [21], who reported that siRNA carrying mismatches at the 3’-end of the sense strand could enhance RNAi activity over conventional

siRNA. Since oligonucleotides containing 4’-thioribonucleosides form thermally more s table duplexes with the natural RNA than natural RNA duplex [10], it could be that thermal stabilization arising from modification with 4’-thioribonucleosides reduces the efficiency of incorporation into RISC. Based on these considerations, we compared the RNAi of matched siRNAs (RNA7 and RNA14) with that of siRNAs containing mismatches on the 3’-end of the sense strand (RNA24 and RNA26). However, we found that RNAi activity was not restored relative to the matched siRNAs. The results of these two types of additional modifications led us to conclude that modifications of siRNAs with 4’-thioribonucleosides do not influence the efficiency of phosphorylation at the 5’-end nor the thermal stability of siRNAs, which would affect the accessibility of helicase to the asymmetric siRNA.

4. Discussion

We have thus shown the first example of RNAi induced by siRNAs modified with 4’-thioribonucleosides in NIH/3T3 cells. These results indicated that the RNAi of siRNAs modified with 4’-thioribonucleosides is dependent on the number and the position of the modifications, especially in the antisense strand. In addition, the central position of the antisense strand was found to be most sensitive to the modification as seen by the comparison of the activities of RNA14–RNA16. This result would appear to be a reasonable assumption because RISC cleaves mRNAs at 10 nucleotides from the 5’-end, i.e., the central position of a 21-nucleotide siRNA [22]. As is commonly recognized, the RNAi machinery is still unclear and quite complex compared with the mechanism of antisense. The gapmer-like mode of modification was ineffective as can be seen in the result of RNA17, and the critical elements for modified siRNAs would seem to lie in other steps, including RISC formation, for example. Since phosphorylation and incorporation of the mismatch of siRNAs did not restore their RNAi activity, other factors should be considered for the modification with 4’-thioribonucleosides–RNAi activity relationship. One possible explanation is the structural change of siRNAs modified with 4’-thioribonucleosides. In our previous paper [10], we reported that not only the 4’-thioRNA:RNA duplex but also the 4’-thioRNA:4’-thioRNA adopts the A-conformation for their mode of base stacking. However, the conformational change of the phosphate–sugar backbone, probably affecting the minor groove of the duplex, arising from the substitution of a furanose ring oxygen by a sulfur atom, was also suggested. This small but critical structural change would affect RISC formation of the modified siRNAs. Chiu et al. [6] suggested that the A-conformation of siRNA is required for RNAi induction, and furthermore, that the structure of the major groove of the siRNA is an essential determinant for RISC formation. Our results may also indicate an importance of the structure of the minor groove for RISC formation. Further investigation of modification–RNAi activity relationship should reveal the structural requirements of RNAi induction.

Thus far, several kinds of modified siRNAs described in the Introduction were under investigation toward therapeutic use. However RNAi activity is altered by not only the mode of modification but also the conditions including the target sequence and the cell lines used, and it is still difficult to determine the best choice of chemical modification of siRNA. Since modifications of siRNAs with 4'-thioribonucleosides improved long-term silencing effect compared with natural siRNA and were well tolerated in RNAi activity compared with 2'-*O*-methyl nucleosides, 4'-thioribonucleosides might have potential for the development of novel and effective chemically modified siRNAs.

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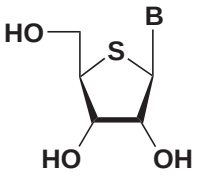
Legends

Fig. 1. Structure of 4’-thioribonucleosides.

Fig. 2. Persistence of silencing of the expression of *Photinus* luciferase gene with siRNA modified with 4’-thioribonucleosides at 25 nM. The data of the RNA1, RNA4, and RNA13 are indicated in gray, red and blue, respectively. Data are averages of at least three independent experiments. Error bars indicate standard deviations. Statistical analyses were carried out by using Student’s *t*-test. Asterisk indicates statistically significant difference ($P < 0.05$) against the data of the RNA1.

Fig. 1.



B = U, C, A, G

Fig. 2.

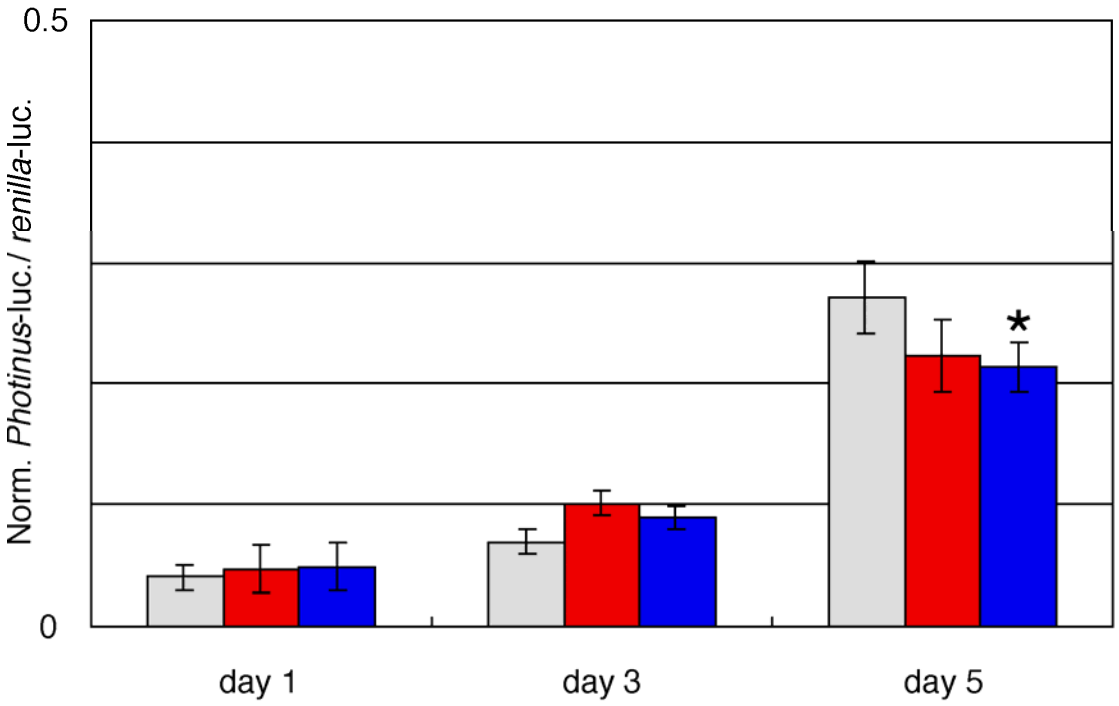


Table 1
Sequence, RNAi activity and IC_{50} of siRNA modified with 4'-thioribonucleosides or 2'-*O*-methyl nucleosides.

name	sequence	RNAi activity (%)	IC_{50} (nM)
scramble	5'-GCGCGCUUUGUAGGAUUCGTT-3' 3'-TTCGCGCGAAACAUCCUAAGC-5'	0±13.3	n.d.
RNA1	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	95.4±0.4	0.10±0.03
RNA2	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	96.3±0.2	n.d.
RNA3	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	93.6±0.4	n.d.
RNA4	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	92.9±0.7	0.12±0.06
RNA5	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	60.3±1.4	n.d.
RNA6	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	79.5±1.3	1.65±1.08
RNA7	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	68.5±4.2	2.09±1.43
RNA8	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	27.5±4.3	n.d.
RNA9	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	16.6±2.3	n.d.
RNA10	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	94.0±0.4	n.d.
RNA11	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	93.8±0.3	n.d.
RNA12	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	87.4±0.4	0.31±0.09
RNA13	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	91.5±1.2	0.16±0.05
RNA14	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	70.9±2.4	2.62±0.70
RNA15	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	72.2±1.5	1.98±1.15
RNA16	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	36.5±7.8	n.d.
RNA17	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	22.8±8.5	n.d.
RNA18	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	94.8±0.6	n.d.
RNA19	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	94.2±1.4	n.d.
RNA20	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	10.3±16.2	n.d.
RNA21	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	30.8±10.5	n.d.

Modifications with 4'-thioribonucleosides are indicated in red.

Modifications with 2'-*O*-methyl nucleosides are indicated in green.

RNAi activity was quantified by the dual luciferase assay and is presented as the inhibition efficiency of target gene (*photinus* luciferase) when cells were treated with 25 nM modified siRNAs.

n.d. means not determined.

Table 2
Sequence, RNAi activity and IC_{50} of siRNA containing two mismatches at the 3'-end of the sense-strand or phosphorylated at the 5'-end of the antisense-strand.

name	sequence	RNAi activity (%)	IC_{50} (nM)
RNA1	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	95.4±0.4	0.10±0.03
RNA7	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	68.5±4.2	2.09±1.43
RNA23	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-p-5'	62.8±3.0	5.30±1.34
RNA24	5'-CGUACGCGGAAUACUUCUCTT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	72.3±1.4	3.32±1.04
RNA14	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	70.9±2.4	2.62±0.70
RNA25	5'-CGUACGCGGAAUACUUCGATT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-p-5'	72.9±7.1	3.39±1.67
RNA26	5'-CGUACGCGGAAUACUUCUCTT-3' 3'-TTGCAUGCGCCUUAUGAAGCU-5'	69.0±1.2	4.84±0.61

Modifications with 4'-thioribonucleosides are indicated in red.

Mismatches are indicated in blue.

RNAi activity was quantified by the dual luciferase assay and is presented as the inhibition efficiency of target gene (*photinus* luciferase) when cells were treated with 25 nM modified siRNAs.