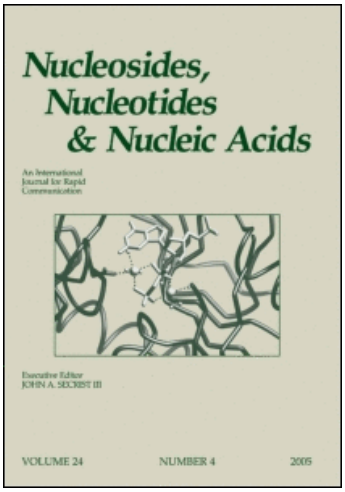

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Prakash, Thazha P. , Naik, Nishant , Sioufi, Namir , Bhat, Balkrishen and Swayze, Eric E.(2009) 'Activity of siRNAs with 2-Thio-2'-*O*-Methyluridine Modification in Mammalian Cells', Nucleosides, Nucleotides and Nucleic Acids, 28: 10, 902 — 910
To link to this Article: DOI: 10.1080/15257770903316145
URL: http://dx.doi.org/10.1080/15257770903316145

Nucleosides, Nucleotides and Nucleic Acids, 28:902–910, 2009

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770903316145

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ACTIVITY OF siRNAs WITH 2-THIO-2′-*O*-METHYLURIDINE MODIFICATION IN MAMMALIAN CELLS

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□ *In a search to identify chemical modifications to improve the properties of siRNA, we have investigated the effect of the 2′-O-methyl-2-thiouridine modification on the biological activity of siRNA. Our results indicate that judicious placement of 2′-O-methyl-2-thiouridine residues could lead to modified siRNA with activity in mammalian cells.*

Keywords siRNA; 2′-*O*-methyl-2-thiouridine; mRNA expression; oligonucleotides

Regulation of mRNA expression using small interfering RNAs (siRNAs) has been recognized as a valuable research tool as well as a novel principle for drug development.[1,2] The success of using short synthetic 19–21 RNA duplexes to inhibit gene expression led to tremendous interest in developing this technology for the potential treatment of various diseases.[3,4]

For effective delivery to therapeutically relevant tissues after systemic administration, chemical modifications are needed to improve the biodistribution and pharmacokinetic properties of the siRNA. Several chemically modified siRNAs have shown improved activity in cell culture as well as in animal models.[3,5,6]

Oligonuleotides containing 2-thiouridine (s^2U) residues are known to favor $C_{3'}$- endo sugar puckering for the nucleotides with an increase in the rigidity of the backbone.[7] They also form thermodynamically stable duplexes with complementary RNA.[8] Furthermore, the increased size and weaker H-bonding ability of sulfur destabilizes a s^2U -G wobble and s^2U-2-amino-A base pairs compared to uridine.[9] As a result, a s^2U containing oligonucleotide provides better selectivity toward binding to complementary oligonucleotides. The RNA duplexes with 2′-*O*-methyl-2-thiouridine (2′-*O*-Me-s^2U) residues exhibited improved *T*m relative to RNA

Received 4 April 2009; accepted 18 August 2009.
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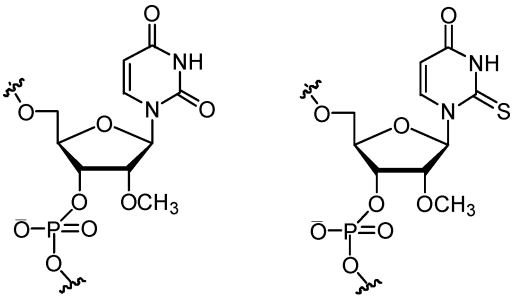
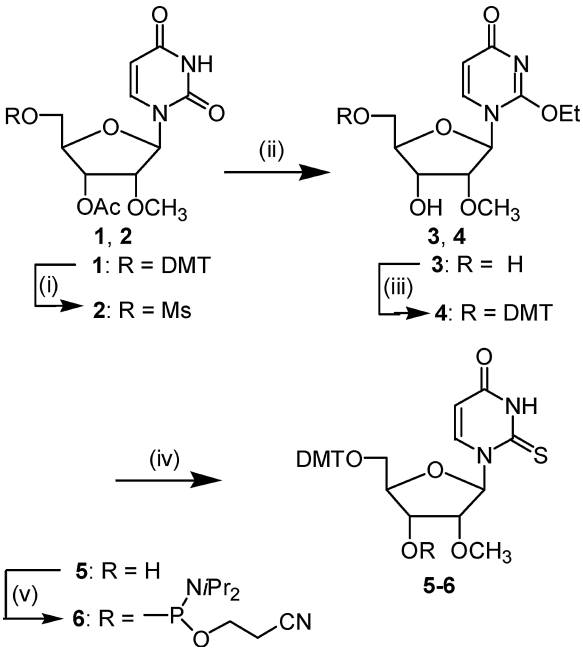


FIGURE 1 2′-*O*-Methyl and 2′-*O*-Me-2-thio modified RNA.

duplex.[10] *T*m of RNA-RNA duplex containing 2′-*O*-Me- s^2U was 5.5°C per modification higher than wild type duplex.[10] The oligonucleotides with 2′-*O*-MOE-2-thiothymidine residues exhibited enhanced binding affinity to target RNA and stability to exonucleases compared to corresponding 2′-*O*-MOE modified oligonucleotides.[11] Recently, it has been reported that s^2U residues suppress recognition of RNA by toll-like receptors.[12]

As a part of our ongoing effort in identifying chemical modifications[6] to improve the properties of siRNA, we have evaluated the 2′-*O*-Me-s^2U modified (Figure 1) siRNA in cell culture. First, we have synthesized 2′-*O*-Me-s^2U-3′-[2-cyanoethyl-*N*,*N*-diisopropyl]phosphoramidite **6** (Scheme 1) according to the reported procedure.[10,11] 3′-*O*-Acetyl-5′-*O*-methanesulfonyl-2′-*O*-methyluridine **2** was prepared from compound **1** according to standard procedures.[11] Compound **2** was dissolved in absolute ethanol and refluxed in the presence of $NaHCO_3$ to yield 2′-*O*-Me-2-*O*-ethyluridine **3** (80%). Compound **3** was stirred with 4,4′-dimethoxytrityl chloride (DMTCl) in anhydrous pyridine to afford the 5′-*O*-DMT derivative **4**. Treatment of compound **4** with H_2S in anhydrous pyridine in presence of 1,1,3,3-tetramethylguanidine (TMG) yielded 5′-*O*-DMT-2′-*O*-Me-s^2U **5** in 82% isolated yield. Compound **5** was phosphitylated at the 3′-position to afford phosphoramidite **6**.

The guide strand (As, Table 1, **7**) of the siRNAs used in this study were designed to target the coding region of human PTEN mRNA.[13] Corresponding guide strands containing 2′-*O*-Me and 2′-*O*-Me-s^2U modifications **10–15** (Table 1) were designed and synthesized. The oligonucleotides **7–15** were synthesized using commercially available 2′-*O*-(*tert*-butyldimethylsilyl) (2′-*O*-TBDMS) protected ribonucleoside phosphoramidites and 2′-*O*-Me modified phosphoramidites on a solid phase oligonucleotide synthesizer according to the reported procedure.[6] The phosphoramidite **6** was used for the incorporation of 2′-*O*-Me-s^2U residues in oligonucleotides **13–15** (Table 1). Oligonucleotides were characterized by mass spectroscopic (ES



SCHEME 1 [a](i) (a) $HOAc/H_2O$ (4:1), room temerpature, (b). methanesulfonyl chloride, pyridine:CH_2Cl_2 (1:1), −20°C; (ii) $NaHCO_3$ (2.5 mol eq.)/EtOH, reflux, 36 hours; (iii) DMT-Cl, pyridine, room temerpature; (iv) H_2S, TMG (10 mol eq.), pyridine, room temperature, 72 hours (v) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, *N*,*N*-diisopropyl ammonium tetrazolide, CH_3CN, room temperature.

MS) analysis (Table 1) and the purity was assessed by high performance liquid chromatography (HPLC).

The siRNA duplexes were formed by combining equivalent molar amount of As and passenger (S, Table 1, **8**) strands mixed and heated at

TABLE 1 2′-*O*-Methyl and 2-thio-2′-*O*-methyl modified RNA used for this study

No.	Sequence[$]	Calcd. mass	Found mass
7	5′ r(UU UGU CUC UGG UCC UUA CUU) 3′	6276.7	6275.8
8	5′ r(AA GUA AGG ACC AGA GAC AAA) 3′	6507.2	6506.1
9	5 ‘r(UU UGU CUC UGG UCC UUA CUU) 3′	6581.9	6580.3
10	5′ r(UU UGU CUC UGG UCC UUA $C^{\nabla}U^{\nabla}U^{\nabla}$) 3′	6544.2	6543.4
11	5′ r(UU UGU CUC $U^{\nabla}G^{\nabla}G^{\nabla}$ UCC UUA CUU) 3′	6544.2	6543.7
12	5′ r(UU UGU $C^{\nabla}U^{\nabla}C^{\nabla}$ UGG UCC UUA CUU) 3′	6544.2	6543.1
13	5′ r(UU UGU CUC UGG UCC UUA $C^{\nabla}U^{*}U^{*}$) 3′	6576.2	6575.2
14	5′ r(UU UGU CUC $U^{*}G^{\nabla}G^{\nabla}$ UCC UUA CUU) 3′	6560.1	6559.2
15	5′ r(UU UGU $C^{\nabla}U^{*}C^{\nabla}$ UGG UCC UUA CUU) 3′	6560.1	6559.2

[$]Backbone chemistry: phosphorothioates, underline represents phosphodiester nucleotides, U^{*} = 2′-*O*-methyl-2-thiouridine, U^{∇} = 2′-*O*-methyluridine, A^{∇} = 2′-*O*-methyl adenosine, C^{∇} = 2′-*O*-methylcytidine, G^{∇} = 2′-*O*-mehtylguanosine.

TABLE 2 Activity of 2-thio-2′-*O*-methyl modified and 2′-*O*-methyl modified siRNA in inhibition of endogenous PTEN mRNA in HeLa cells

siRNA	As 5′-3′/S 3′-5′	IC_{50} nM
7:8	5′ r(UUUGUCUCUGGUCCUUACUU) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	0.2
9:8	5′ r(UUUGUCUCUGGUCCUUACUU) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	1.8
10:8	5′ r(UUUGUCUCUGGUCCUUAC^{∇} U^{∇} U^{∇}) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	4.3
11:8	5′ r(UUUGUCUCU^{∇} G^{∇} G^{∇}UCCUUACUU) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	2.1
12:8	5′ r(UUUGUC^{∇} U^{∇} C^{∇}UGGUCCUUACUU) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	1.2
13:8	5′ r(UUUGUCUCUGGUCCUUAC^{∇} U^{*} U^{*}) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	4.6
14:8	5′ r(UUUGUCUCU^{*} G^{∇} G^{∇}UCCUUACUU) 3′	7.1
15:8	3′ r(AAACAGAGACCAGGAAUGAA) 5′5′ r(UUUGUC^{∇} U^{*} C^{∇}UGGUCCUUACUU) 3′ 3′ r(AAACAGAGACCAGGAAUGAA) 5′	2.5

S = passenger strand; As = guide strand. Underline indicate phosphodiester linkages. U^{*} = 2′-*O*-methyl-2-thiouridine, U^{∇} = 2′-*O*-methyluridine A^{∇} = 2′-*O*-methyladenosine, C^{∇} = 2′-*O*-methylcytidine, G^{∇} = 2′-*O*-mehtylguanosine.

90°C for 1 minute. The solution was incubated at 37°C for 6 hours. The percentage of the duplex formed was determined using CGE analysis.[6] Synthetic siRNA duplexes were transfected into cells using Lipofectin reagent. Cells were harvested 18–24 hours posttransfection and total cellular RNA was isolated on an RNeasy 3000 BioRobot (Qiagen, Valencia, CA, USA). Reduction of target mRNA expression was determined by real time quantitative RT-PCR. Total RNA for each well was measured using RiboGreen[6] (Molecular Probes, Eugene, OR, USA), and these values were used for sample-to-sample normalization.

The siRNA duplex **9:8** (Table 2) with phosphorothioate (PS) linkages on the guide strand, exhibited dose dependent inhibition of PTEN mRNA expression in cell culture (IC_{50} 1.8 nM, Table 2). The siRNA duplex **7:8** (Table 2, IC_{50} 0.2 nM) with the phosphodiester backbone chemistry (PO) on the guide strand, showed 9 fold better activity.[6b] In this study, we have used As strands with PS and S strand with PO linkages, respectively.

In our earlier report, we have evaluated the positional preference of 2′-*O*-Me modification on the activity of siRNA.[6b] The siRNA with three 2′-*O*-Me modified residues at the 3′-end of the As strand was more active than siRNA with three 2′-*O*-Me modification at the 5′ end of the As strand.[6b] The activity of the siRNAs with and without 2′-*O*-Me modifications in the S strands was similar.[6bb] Here we have evaluated the effect of replacing 2′-*O*-Me U with 2′-*O*-Me-s^2U on cellular activity of siRNA. In general, replacing

2′-*O*-Me-U with 2′-*O*-Me-s^{2}U residue is tolerated in the As strand. siRNA duplex **10**:**8** (IC_{50} 4.3 nM, Table 2) containing 2′-*O*-Me U residues and **13**:**8** containing 2′-*O*-Me-s^{2}U (IC_{50} 4.6 nM, Table 2) residues at the 3′-end of the As strand exhibited similar potency. However, when the 2′-*O*-Me-s^{2}U was placed in the middle of the As strand (Table 2, **14**:**8** IC_{50} 7.1 nM, **15**:**8**, IC_{50} 2.5 nM), the activity was less than the corresponding 2′-*O*-Me U siRNA (Table 2, **11**:**8** IC_{50} 2.1 nM and **12**:**8** IC_{50} 1.2 nM). These data suggest that 2′-*O*-Me-s^{2}U is well tolerated at the 3′-end of the As strand and 2-thio base modification could maintain the activity of siRNA containing tandem 2′-*O*-Me modifications.

In conclusion, we have designed and synthesized siRNA duplexes with 2′-*O*-Me-s^{2}U residues in the As strand. 2-Thio base modification in combination with 2′-*O*-Me sugar modification was well tolerated at the 3′-end of the As strand of siRNA. The known nuclease resistant properties[11] of 2-thio modification in conjunction with 2′-sugar modification, and the reported results of 2-thiouridine suppress RNA recognition by toll-like recepotors[12] suggests that this could be a useful modification for siRNA therapeutics when used in combination with other modifications in the appropriate substitution pattern.

EXPERIMENTAL

General

Reagents and phosphoramidites for oligonucleotide synthesis were purchased from Glen Research, Inc. (Sterling, VA, USA). All other reagents and anhydrous solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. ^{1}H NMR spectra were referenced using internal standard $(CH_3)_4Si$ and ^{31}P NMR spectra were referenced using external standard 85% H_3PO_4. Mass spectra were recorded by College of Chemistry, University of California (Berkeley, CA, USA).

5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-methyl-2-thiouridine 5

Compound **4** (2.72 g, 4.38 mmol) was placed in a RB flask under an argon atmosphere and cooled in a freezing bath. A solution of anhydrous TMG (2.93 mL, 23.25 mmol) in pyridine (57 mL) was flushed with an argon and cooled to 0°C in a freezing bath and solution was saturated with hydrogen sulfide for 45 minutes. The solution was then transferred into the precooled flask containing compound **5** under an argon pressure. The temperature of the flask was slowly brought up to room temperature and stored for 72 hours. H_2S was gently flushed out into a Clorox bath and then pyridine was removed from the reaction mixture under vacuum. The residue suspended in ethyl acetate was subjected to water wash followed

by standard workup. The desired product was purified by flash silica gel column chromatography using ethyl acetate and hexane (1:1) as eluent to yield compound **5** as a white foam (2.04 g, 82%): ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.33–3.49 (m, 2H), 3.51 (s, 3H) 3.73 (s, 6H), 3.80–3.85 (m, 1H), 4.01 (m, 1H), 4.30 (q, J = 7.6, 4.8, 7.4 Hz, 1H), 5.29 (d, J = 7.2 Hz, 1H), 5.39 (d, J = 8.2 Hz, 1H), 6.52 (d, J = 2.0 Hz, 1H), 6.91 (d, J = 8.8 Hz, 4H), 7.22–7.41 (m, 9H), 7.99 (d, J = 8.2 Hz, 1H), 12.71 (s, 1H); HRMS (FAB) *m/z* calcd for $C_{31}H_{33}N_2O_7S^+$ 577.2007, found 577.2008.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(methyl)-2-thiouridine-3′-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite 6

Compound **5** (1.14 g, 1.98 mmol) was mixed with tetrazole diisopropylammonium salt (0.17 g, 0.99 mmol) and dried over anhydrous P_2O_5 under reduced pressure overnight. The mixture was suspended in anhydrous acetonitrile (7 mL) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (1.26 mL, 3.98 mmol) was added. The reaction mixture was stirred at the ambient temperature for 7 hours under an argon atmosphere. The solvent was removed under reduced pressure and the residue obtained was dissolved in ethyl acetate (20 mL), washed with saturated aqueous sodium bicarbonate followed by standard workup. The residue was purified by flash silica gel column chromatography (eluent: ethyl acetate / hexane, 1:1) to yield compound **6** (1.11 g, 65%). ^{31}P NMR (80 MHz, $CDCl_3$) δ 151.36; HRMS (FAB) *m/z* calcd for $C_{40}H_{50}N_4O_8PS^+$ 777.3087, found 777.3098.

Synthesis of Oligonucleotides 7–15

The standard phosphoramidites and solid supports were used for incorporation of A, U, G, and C residues. A 0.1 M solution of the phosphoramidites in anhydrous acetonitrile was used for the synthesis. The modified oligonucleotides were synthesized on universal solid support.[14,15] A 0.1 M solution of phosphoramidite **6** was used for the incorporation of 2-*O*-Me-s^{2}U residues. Twelve equivalents of phosphoramidite solutions were delivered in two portions, each followed by a 6 minutes coupling wait time. All other steps in the protocol supplied by the manufacturer were used without modification. 0.2 M PADS in 1:1 3-picoline/CH_3CN were used as a sulfurization reagent with 3 minutes contact time.[16] A solution of *tert*-butyl hydroperoxide/acetonitrile/water (10:87:3) was used to oxidize inter nucleosidic phosphite to phosphate. The step-wise coupling efficiencies were more than 97%. After completion of the synthesis, the solid support bearing oligonucleotide was suspended in aqueous ammonia (28–30 wt%): ethanol (3: 1) and heated at 55°C for 6 hours to complete the removal of all

protecting groups except TBDMS group at 2′-position. The solid support was filtered and the filtrate was concentrated to dryness. The residue obtained was resuspended in triethylamine trihydrofluoride/triethylamine/1-methyl-2-pyrrolidinone solution (0.75 mL of a solution of 1 ml of triethylamine trihydrofluoride, 750 μl triethylamine and 1.5 mL 1-methyl-2-pyrrolidine, to provide a 1.4 M HF concentration) and heated at 65°C for 1.5 hours to remove the TBDMS groups at the 2′-position. The reaction was quenched with 1.5 M ammonium bicarbonate (0.75 mL) diluted with DNase/RNase free water and purified by HPLC on a strong anion exchange column (Mono Q, Pharmacia Biotech, 16/10, 20 mL, 10 μm, ionic capacity 0.27–0.37 mmol mL^{-1}, A = 100 mM ammonium acetate, 30% aqueous acetonitrile, B = 1.5 M NaBr in A, 0–60% B in 40 minutes, flow 1.5 mL min^{-1}, λ = 260 nm). Fractions containing full-length oligonucleotides were pooled together (assessed by LC MS analysis >90%) and evaporated. The residue was desalted by HPLC on a reverse phase column (HPLC, C-18 column, Waters, 7.8 × 300 mm, A = 100 mM ammonium acetate, B = acetonitrile, C = water, 100% A two column volume, 100% C two column volume, eluted oligonuclotide with 50% B in water, flow 2.5 mL min^{-1}, λ 260 nm). The oligonucleotides were characterized by ES MS and purity was assessed by HPLC (Waters, C-18, 3.9 × 300 mm, A = 100 mM triethylammonium acetate, pH = 7, B = acetonitrile, 5 to 60% B in 40 min, flow 1.5 mL min^{-1}, λ = 260 nm).

siRNA Preparation

The siRNA duplexes were prepared according to the reported procedure.[6]

Cell Culture and Transfection of Cells

HeLa cells (American Type Tissue Culture Collection, Manassas, VA, USA) were cultured in culture flask in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, CA, USA), liquid (high glucose) supplemented with 10% heat inactivated fetal bovine serum (FBS). The cells were not allowed to exceed 75–80% confluency. Prior to treatment (24 h before treatment), the cells were detached from the flask using Trypsin (Invitrogen) and plated in 96-well plates at a density of 5,000 cells/well. The cells were transfected with siRNAs complexed with 6 μg mL^{-1} Lipofectin (Invitrogen) in serum-free Opti-MEM I Reduced-Serum Medium. The cells were incubated in the transfection medium for 4 hours, and transfection medium was removed from the cells and replaced with fresh DMEM, 10% fetal calf serum and incubated at 37°C, 5% CO_2 for 16 hours.

RNA Expression Analysis

Total RNA was harvested after 16 hours using the RNeasy process from Qiagen according to the manufacturer's protocol. Gene expression was determined via real time quantitative RT-PCR on the ABI Prism 7900 system (Applied Biosystems, Foster City, CA, USA) as described in the literature.[17–18] The following primer probe set (Qiagen) was used: hu PTEN (Accession No. U92436.1), forward primer 5′-AATGGCTAAGTGAAGATGACAATCAT, reverse primer 5′- TGCACATATCATTACACCAGTTCGT and FAM/TAMRA probe 5′-TTGCAGCAATTCACTGTAAAGCTGGAAAGG. Total RNA for each well was measured using RiboGreen (Molecular Probes), and these values were used for sample-to-sample normalization.[19] IC_{50} values were calculated from the linear regression analysis from the plot of the logarithmic value of siRNA concentration versus percentage of untreated control.

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