RNA Interference in Mammalian Cells by Chemically-Modified RNA†

Dwaine A. Braasch,‡ Susan Jensen,§ Yinghui Liu,‡ Kiran Kaur,|| Khalil Arar,⊥ Michael A. White,|| and David R. Corey*,‡

Departments of Pharmacology and Biochemistry, The Center for Biomedical Inventions, and the Department of Cell Biology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, and Proligo LLC, 1, Rue Delaunay, Paris, 75011 Paris, France

Received March 7, 2003; Revised Manuscript Received May 1, 2003

ABSTRACT: RNA interference (RNAi) is proving to be a robust and versatile technique for controlling gene expression in mammalian cells. To fully realize its potential in vivo, however, it may be necessary to introduce chemical modifications to optimize potency, stability, and pharmacokinetic properties. Here, we test the effects of chemical modifications on RNA stability and inhibition of gene expression. We find that RNA duplexes containing either phosphodiester or varying numbers of phosphorothioate linkages are remarkably stable during prolonged incubations in serum. Treatment of cells with RNA duplexes containing phosphorothioate linkages leads to selective inhibition of gene expression. RNAi also tolerates the introduction of 2′-deoxy-2′-fluorouridine or locked nucleic acid (LNA) nucleotides. Introduction of LNA nucleotides also substantially increases the thermal stability of modified RNA duplexes without compromising the efficiency of RNAi. These results suggest that inhibition of gene expression by RNAi is compatible with a broad spectrum of chemical modifications to the duplex, affording a wide range of useful options for probing the mechanism of RNAi and for improving RNA interference in vivo.

The control of gene expression with nucleic acids is a powerful tool for investigating protein function inside cells and may provide a new class of therapeutics (*1*−*4*). Short interfering RNAs (siRNAs)[1] can regulate gene expression in mammalian cells through RNA interference (RNAi) (*5*−*10*). It is important that siRNAs be compared to chemically optimized classical antisense approaches (i.e., approaches that employ single-stranded oligonucleotides to target mRNA), but initial results suggest that (i) fewer RNA duplexes may need to be screened to identify active siRNAs and (ii) siRNAs may be tend to be more potent inhibitors of gene expression and less toxic to cells, making them more robust tools for many investigators. Reports also indicate that siRNA can function in vivo (*11*−*14*).

Chemical modifications have been essential for the development of classical antisense oligonucleotides and their advancement into clinical trials (*1*−*4*, *15*). The two strands of the siRNA duplex can be produced by standard protocols, and many of the chemical modifications that have been developed to improve classical antisense oligonucleotides can also be introduced into RNA. Because these modifications have been critical for the development of classical antisense oligonucleotides, they may improve the thermal stability, serum stability, cellular activity, or pharmacokinetic properties of siRNA. Chemical modifications should be useful tools for investigations into the mechanism of RNAi and the function of the proteins that make up the RNAi induced silencing complex (RISC).

One important chemical modification for classical antisense oligonucleotides is the replacement of phosphodiester with phosphorothioate (PS) linkages (*16*−*18*). PS linkages reduce cleavage by nucleases and increase the half-life of oligonucleotides in vivo. PS linkages also increase binding to serum proteins leading to reduced rates of clearance and improved bioavailability of antisense oligomers (*18*).

Another widely used class of alteration involves modification of the 2′ position of the ribose ring, including 2′-*O*-methyl (2′-*O*-meRNA) (*19*), 2′-deoxy-2′-fluorouridine (*20*, *21*), and locked nucleic acid (LNA) (*22*−*24*), which contains a methylene linkage between the 2′ and the 4′ positions of the ribose (Figure 1). These chemical modifications can increase stability to degradation by nucleases or improve thermal stability as measured by T_m values. None of these modifications support RNAse H activity. Therefore, oligomers that contain LNA, 2′-*O*-meRNA, or 2′-deoxy-2′-fluorouridine bases must also contain several consecutive DNA bases if cleavage of RNA by RNAse H is desired. Another potential advantage for using chemical modifications is that elimination of the 2′-hydroxyl simplifies synthesis, deprotection, and purification protocols and may assist development of methods for obtaining the large amounts of siRNA needed for therapeutic applications.

In *Caenorhabditis elegans*, Fire and co-workers report that duplex RNA that is partially substituted with PS linkages on one or both strands continues to support RNA interference (*25*). They also observed that substitution with 2′-deoxy-2′-

† This work was supported by grants from the National Institutes of Health (GM 60624 to D.R.C.) and the Robert A. Welch Foundation (I-1244 to D.R.C. and I-1414 to M.A.W.)

* Corresponding author. Phone: (214) 648-5096. Fax: (214) 648-5095. E-mail: david.corey@utsouthwestern.edu.

‡ Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center at Dallas.

§ The Center for Biomedical Inventions, University of Texas Southwestern Medical Center at Dallas.

|| Department of Cell Biology,University of Texas Southwestern Medical Center at Dallas.

⊥ Proligo LLC.

[1] Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA; PO, phosphodiester; PS, phosphorothioate; hCav, human caveolin; LNA, locked nucleic acid; RISC, RNA induced silencing complex; T_m, melting temperature.

10.1021/bi0343774 CCC: $25.00
Published on Web 06/11/2003

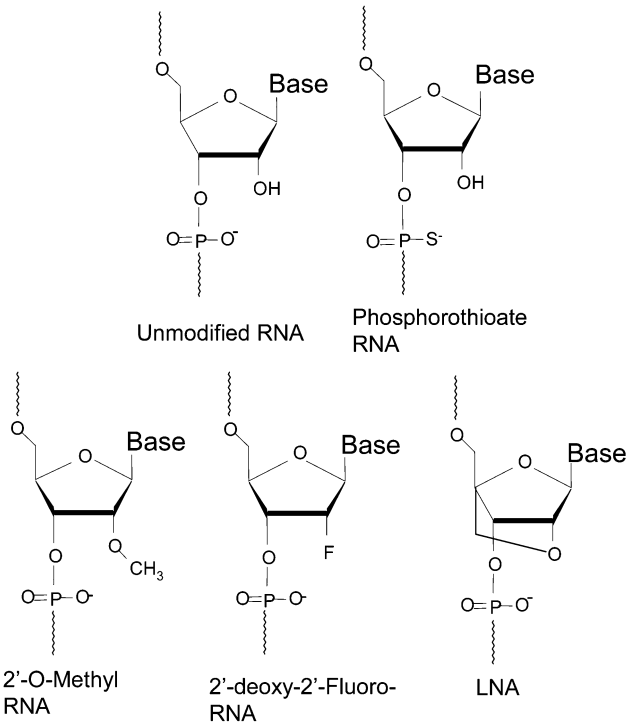


FIGURE 1: Chemical structures of native RNA and the chemical modifications used in these studies. Clockwise from top: unmodified RNA, phosphorothioate (PS) RNA, 2′-*O*-methyl RNA, 2′-deoxy-2′-fluorouridine, and locked nucleic acid (LNA).

fluorouridine also did not affect RNAi (*25*). In experiments in *Drosophila* cell lysate, Tuschl and co-workers have shown that siRNA containing 2′-*O*-methyl modifications to either the sense or the antisense strands are inactive (*26*). In mammalian cell culture, Zamore and co-workers found that removal of the 3′-hydroxyl does not affect activity (*27*), while Rana and Chiu found that free 5′-hydroxyl groups are essential for activity and observed that a blocked 3′-terminus was tolerated (*28*). Prydz and co-workers have reported that minimal substitution with 2′-*O*-methyl nucleotides or PS linkages is tolerated but that extensive PS substitution is toxic to cells (*29*).

Here, we examine the effect of chemical modifications on RNAi in mammalian cell cultures. We observe that unmodified duplex RNA is remarkably stable in serum. Inhibition of gene expression is compatible with PS, 2′-deoxy-2′-fluorouridine, or LNA modifications. Introduction of LNA nucleotides can also greatly increase thermal stability. These results suggest that chemical modifications can provide useful tools for analyzing the mechanism of mammalian RNAi and for improving the efficacy of RNAi in mammalian cell culture and in animals.

MATERIALS AND METHODS

RNA Oligonucleotides. Oligomers were synthesized on a 0.2 μmol scale and were obtained after lyophilization from Oligos Ect. (Wilsonville, OR) or from the RNA synthesis core at the Center for Biomedical Inventions (University of Texas Southwestern Medical Center at Dallas). Oligomers that contained LNA nucleotides were kindly provided by Proligo LLC (Paris, France).

RNA oligomers containing 2′-deoxy-2′-fluorouridine (Glen Research, Sterling, VA) were synthesized on an ABI 392 synthesizer using either dT columns or on Universal Support II columns (Glen Research) for oligomers containing a 2′-fluoruridine at the terminal 3′ position. To aid purification, the 5′-terminal group dimethoxytrityl group was retained after synthesis. 2′-Deoxy-2′-fluorouridine phosphoramidate was dissolved in 3.9 mL of acetonitrile. All other reagents including deblock (dichloroacetic acid), activator (0.25 M 5-ethylthio-1*H*-tetrazole in acetonitrile), Cap A (THF/lutidine/acetic anhydride), Cap B (10% *N*-methylimidazole in tetrahydrofuran), and oxidizer (0.1 M iodine in THF, water, pyridine) were obtained from Transgenomics Inc. (Wayne, PA). Phosphorothioate linkages were introduced using 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Glen Research) as oxidizer. RNA nucleotides were protected with *tert*-butyl dimethyl silyl groups (TBDMS). Final deprotection was performed using manufacturer recommendations for both types of supports used.

Dimethoxytrityl (DMT)-protected oligomers were desalted by dissolving the RNA oligomer in 1 mL of 0.1 M triethylammonium acetate (TEAA). A Poly-Pak cartridge (Glen Research) was preconditioned by washing with 2 mL of acetonitrile, followed by 2 mL of 2 M TEAA. The RNA oligomer was loaded onto the cartridge, and the filtrate was collected and reloaded twice. The bound oligomer was washed with 3 × 10 mL of 0.3 M ammonium acetate, followed by 4 × 10 mL of sterile warm (70 °C) water. The cartridge was washed with 4 mL of a 2% trifluoroacetic acid (in water) solution with incubations at room temperature for 3 min. This incubation removes the DMT group. The cartridges were then immediately flushed with 10 mL of water followed by 3 × 10 mL of 0.3 M ammonium acetate followed by 4 × 10 mL of warm water. Oligonucleotides were eluted with 1.5 mL of 50% aqueous acetonitrile in an RNase-free vial and dried at 65 °C with a gentle nitrogen flow. Stock solutions were prepared using diethylpyrocarbonate (DEPC) treated sterile water. Oligonucleotides were allowed to sit at room temperature for 5 min, vortexed several times briefly (2−4 s), then heated to at least 70 °C to enhance solubility, and centrifuged briefly to remove remaining insoluble material. Concentrations were determined by diluting the stock solutions in DEPC-treated water followed by analysis using a UV spectrometer. Concentrations were calculated using a 40 ng/μL conversion per 1 optical density unit at 260 nm.

Duplex Formation. RNA duplexes (100 μM each) were prepared by the annealing of complementary oligonucleotides in thin-walled PCR tubes containing 2.5 × PBS (10X phosphate buffered saline, pH 7.4, without calcium or magnesium chloride, 10 mM KH_2PO_4, 1550 mM NaCl, 30 mM Na_2HPO_4-$7H_2O$, Invitrogen). Annealing of RNA duplexes was performed in a thermal cycler according to the following temperature profile. Reductions in temperatures occurred in 1 min with hold times indicated (°C, min): 95, 5; 85, 1; 75, 1; 65, 5; 55, 1; 45, 1; 35, 5; 25, 1; and 15 °C, 1 min, hold at 15 °C. After annealing, the RNA duplexes were stored at −20 °C.

Melting Temperature Analysis. Melting temperature (T_m) studies were performed by measuring the change in absorbance at 260 nm using a Cary 100 Bio UV/Vis Spectrophotometer equipped with a 12-position sample holder and a Peltier temperature controller (Varian Inc., Walnut Creek, CA). Determinations were performed in a 0.9 mL stoppered

semimicro quartz cuvette (Varian). The sample was prepared by mixing 8 μL of 100 μM annealed RNA duplex stock solution with 398 μL of 0.1 M diammonium citrate buffer, pH 7.0. Diammonium citrate was used because it produced the clearest thermal transitions for the fully PS-substituted duplexes and was used for all measurements to ensure consistency. Samples were overlaid with 400 μL of mineral oil to prevent evaporation at higher temperatures and to make the upper and lower baselines more consistent. Data were collected with the Cary WinUV Thermal software from 98 to 14 °C and from 14 to 98 °C in 2 °C increments with an equilibration time of 0.2 min at each temperature after an initial 2 min equilibration prior to starting the temperature ramping. Data were collected in both directions (denaturation and annealing) to confirm that the observed curves were reversible. Data were subjected to nonlinear curve fit analysis, and the T_m was determined using van't Hoff parameters included in the software. Independent analyses were performed for the data corresponding to the denaturation and annealing profiles, and the average value was reported.

Transfection of siRNA into HeLa Cells with Cationic Lipid. HeLa cells (ATCC CCL-2) were cultured in Dulbeco's Modified Eagles Medium (DMEM) containing 4 g/L glucose (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 20 mM HEPES buffer (final concentration, pH 7.4), 4 mM L-glutamine, 500 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.01 mg/mL tylosin (anti-mycoplasma reagent) (Sigma, St. Louis, MO). Cells were cultured at 37 °C with a 5.2% CO_2 atmosphere.

Cells were seeded in 24-well plates at a density of 18 000−20 000 cells per well and allowed to attach to the culture vessel for 8−12 h prior to transfection. Duplex siRNA were prepared as described above at stock concentrations of either 20 μM or 100 μM. Lipid-siRNA complexes were prepared at 200 nM, and serial dilutions were performed after formation of siRNA-lipid complexes. OligofectAMINE (Invitrogen, Carlsbad, CA) was used to deliver siRNAs. Transfection solutions were prepared according to manufacturer protocols with antibiotic free medium, and 400 μL of the transfection solution was dispensed per well. Transfections were conducted overnight (~10−14 h), after which the solution was removed and replaced with growth medium containing antibiotics. Cells were harvested for Western blot analysis 48-h post-transfection (this was the time required for cells to reach confluence within the wells).

Western Blot Analysis for Human Caveolin. Cells were harvested by washing the cells once with 1X PBS, aspirated, and treated with 50 μL of a trypsin solution (0.05% Trypsin, 0.53 mM EDTA·4Na, Invitrogen) at 37 °C for 2 min. Trypsin was inactivated by the addition of culture medium containing 20% FBS. The contents of each well were transferred separately into 1.5 mL microfuge tubes and centrifuged at 1000*g* for 5 min at 4 °C. The supernatant fluid was discarded, and the cell pellet was suspended in 400 μL of 1X PBS. The contents were again centrifuged at 1000*g* for 5 min at 4 °C. Cells were then lysed with 40−50 μL of ice-cold lysis buffer (120 mM Tris-base, pH 7.4, 120 mM NaCl, 1 mM Na_2-EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.1 mM sodium fluoride, 0.1 mM sodium vanadate, 0.5% v/v Nonidet P-40) containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Tubes were vortexed for 10−20 s with short bursts and then allowed to incubate for 5−10 min at room temperature. Samples that were used immediately were centrifuged at 12 000*g* for 5 min at 4 °C to pellet debris. Samples not used for immediate analysis were stored at 4 or −20 °C.

Protein content was determined for each sample in a 96-well plate format by the BCA method (Pierce, Rockford, IL). Western analysis by SDS−PAGE was performed using standard methods. Primary antibodies rabbit polyclonal anti-human caveolin (BD Transduction Laboratories, San Jose, CA) were diluted 1:5000 in TTBS and placed on a rocker platform for 1 h at room temperature. The membranes were washed twice for 5 min each in TTBS. Secondary antibody conjugate (HRP conjugated goat-anti-rabbit or goat anti-mouse) were diluted 1:12 500 in TTBS and placed on a rocker platform for 1 h at room temperature. Membranes were then washed four times for 5 min each in TTBS. Each membrane was incubated for 5 min in 4 mL of SuperSignal West Pico Chemiluminescent substrate (Pierce), then drained, placed in a transparent sheet protector, exposed to BioMax Light film (Eastman Kodak Company, Rochester, NY) for 1−60 s, and developed according to manufacturer's recommendations. Films were quantified from the scanned images using Sigma Gel analysis software (SPSS Science, Chicago, IL). The control antibody was mouse anti-α-tubulin (clone B-5-1-2) (Sigma).

Assays for Serum Stability. A 15 μL aliquot of duplex RNA (500 ng/μL each strand) or single-strand RNA (1000 ng/μL) added to 15 μL of complete tissue culture medium was added, resulting in a solution containing 5% final concentration of fetal bovine serum. At specified times, a 5 μL aliquot was removed, mixed with 10 μL of formamide, flash frozen at −70 °C, and stored at −20 °C. Samples were analyzed using a 15% polyacrylamide gel (National Diagnostics) containing 7 M urea and TBE (0.089 M Tris base, 0.089 M boric acid (pH 8.3), and 2mM Na_2EDTA) buffer. Following electrophoresis, gels were stained for 40 min in a 1:10 000 dilution of SYBR Green II RNA gel stain (Molecular Probes, Eugene, OR) prepared in DEPC treated water and visualized on a UV-transilluminator. The gel images were obtained using an IS-500 Gel Documentation System (Alpha Innotech, San Leandro, CA).

RT-PCR Analysis. HeLa cells were transfected as above with 200 nM inhibitor. Cells were harvested by washing the cells once with 1X PBS, aspirated, and treated with 100 μL of a trypsin solution (0.05% Trypsin, 0.53 mM EDTA·4Na, Invitrogen) at 37 °C for 2 min. Trypsin was inactivated by the addition of 400 μL of culture medium containing 20% FBS. The contents of each well were transferred separately into 1.5 mL microfuge tubes and centrifuged at 1000*g* for 5 min at 4 °C. The supernatant fluid was discarded, and the cell pellet was suspended in 400 μL of 1X PBS. The contents were again centrifuged at 1000*g* for 5 min at 4 °C. The supernatant fluid was discarded, and the cell pellet was flash frozen in an isopropyl alcohol−dry ice bath for 20 min and stored at −80 °C.

Cell pellets were slowly thawed on ice, and the total RNA was isolated using the RNAqueous procedure (Ambion Inc., Austin, TX). RNA was eluted from the binding matrix, with a 60 and a 40 μL aliquot of elution buffer preheated to 80 °C. The concentration of total RNA as determined spectrophotometrically (A_{260} × 40 ng/μL × inv. dilution). RNA

samples were frozen and stored at −20 °C short term, until RT-PCR was performed.

RT-PCR primers were designed for human caveolin-1 and cyclophillin C based upon the GenBank accession numbers NM_001753 and NM_000943, respectively. Primer set one for caveolin-1 was designed to be completely contained within the coding region flanking the siRNA target site (+91...+110), while primer set two was intended to amplify the complete transcript including portions of the 5′-UTR and 3′-UTR regions. The sequences and mRNA target sites for the primer sets were hCav sense primer-1 5′-TGTCTGGGGGCAAATACG-3′ (+2 to +19); hCav antisense primer-1 5′-CCAAAGAGGGCAGACAGC-3′ (+322 to +305); hCav sense primer-2 5′-CCCCATACAATACAAGAT-3′ (−91 to −71); hCav antisense primer-2 5′-ATCAGGTATACTTCTATCCTTGA-3′ (+567 to +545); cyclophilin sense primer 5′-CTGGTTGGACGGCAAACAT-3′ (+462 to +468); and cyclophilin antisense primer 5′-AGGCGTTTTCACGTCTATCTTG-3′ (+612 to +591).

RT-PCR was performed using SuperScript II One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) according to manufacturer's protocol adjusted for a total reaction mixture of 25 μL containing 500 ng of total RNA template per 200 μL of reaction tube. Reverse transcriptase reactions were performed with a PX2 thermal cycler (Thermo Hybaid, Middlesex, UK) for 30 min at 50 °C. The reverse transcriptase was inactivated commensurate with Taq polymerase activation with a 2 min incubation at 94 °C. Samples were subsequently amplified by PCR for 20 cycles (94 °C, 15 s; 45 °C, 30 s; 72 °C, 30 s). At the conclusion of amplification, all products were extended to full length with a 5 min incubation at 72 °C and visualized with CybrGreen II (Molecular Probes). Quantification was performed based on area integration of stained amplification products using Sigma Gel Software (SPSS Inc., Chicago, IL).

Fluorescence Microscopy. Sense strands PO-RNA and PS-RNA were synthesized to include a Cy3 fluorescent label at the 5′-terminus. The labeled sense strand oligomers were annealed to their antisense complement of same chemistry and delivered into HeLa cells as described above into a Lab-Tek 4-well chambered coverglass (Nalgene Nunc International, Naperville, IL). Transfection of duplex RNAs were performed at 100 nM in 400 μL with OligofectAMINE. After an overnight transfection, cells were washed 2× with 500 μL of Opti-MEM at room temperature with a 5 min room-temperature incubation between washes. Cells were then incubated for 30 min in a 50% (v/v) solution of Opti-MEM and PBS containing 0.05 mg/mL Hoechst 33258 stain (Sigma) and then washed 5× with 500 μL of Opti-MEM. After the last wash, slides were analyzed using a Zeiss Axiovert 200 M inverted transmitted light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a digital imaging system and Slidebook imaging software (Intelligent Imaging Innovations, Inc., Denver, CO). Confocal microscopy images of HeLa cells treated with Hoechst 33258 and sense strand Cy3-labeled RNA annealed to an antisense strand. Cells were imaged at 20× using Cy3 and UV filter sets to image Cy3 and Hoechst, respectively.

RESULTS AND DISCUSSION

Experimental Design. These studies investigate siRNA duplexes that contain chemical modifications including phosphorothioate (PS) linkages between the nucleotides, 2′-*O*-methyl (2′-*O*-Me) (*19*), 2′-deoxy-2′-fluorouridine (*20*, *21*), and locked nucleic acid (LNA) (*22*−*24*) modifications to the ribose (Figure 1). Each of these modifications can be introduced into RNA without substantial alteration of standard synthesis protocols. Purity was evaluated by gel electrophoresis, and oligomers were desalted to remove small molecule contaminants that might increase nonsequence-specific toxicity to cultured cells.

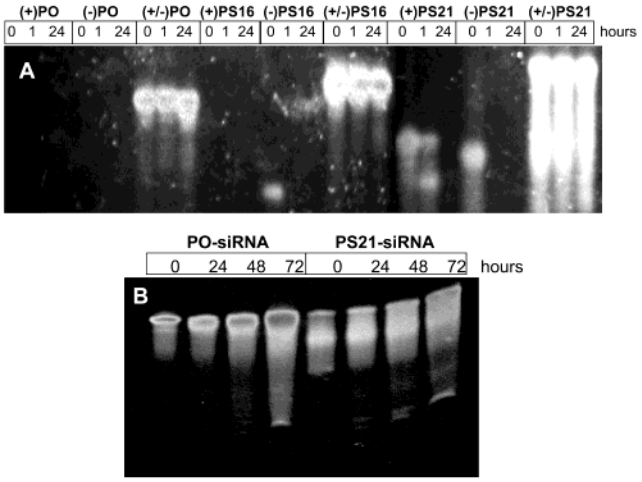


FIGURE 2: (A) Serum stability of single- and double-stranded PO and PS RNAs. The PS RNAs contained either 16 (hCav PS16) or 21 PS (hCav PS21) substitutions on each strand of the duplex. Incubations were performed for 0, 1, or 24 h. The degradation of RNA that occurs at the $t = 0$ incubations occurs during the mixing (~30 s) of RNA and serum prior to freezing of sample. (−) = Single-stranded antisense RNA. (+) = Single-stranded sense RNA. (±) = Duplex RNA. (B) Serum stability of double-stranded (±) PO and PS RNA (hCav PS21) over 0, 24, 48, and 72 h at 37 °C.

We use several assays to test the properties of siRNA containing chemical modifications. We first investigated stability to nuclease digestion by incubation in serum and then used fluorescently labeled duplex RNAs to investigate uptake by cells. Once stability and uptake were established, we examined inhibition of protein expression. As a target we chose the mRNA for an endogenously expressed gene, human caveolin-1 (hCav). hCav is a 23 kD protein that forms caveolae, a flask shaped invagination of the plasma membrane that has been implicated in several cellular signaling processes (*30*, *31*). Reduced expression of hCav in cells upon addition of siRNA has been demonstrated previously (*32*) and provided a useful starting point for our investigations. In all experiments, expression of α-tubulin was used as a loading control.

Stability of Duplex RNA in Serum. To be active inside cells a nucleic acid must be stable to digestion by nucleases, and to test the stability of RNAs used in these studies, we incubated RNA single or double strands in complete cell culture medium containing 5% fetal bovine serum at 37 °C (Figure 2; see Figure 4 for sequences). Not surprisingly, single-stranded PO-RNA was completely degraded during incubations that were as short as 30 s. We were surprised, however, to note that duplex PO-RNA was stable during incubations in serum for up to 72 h (Figure 2A,B). Similar results were observed for PO-RNA duplexes complementary to firefly luciferase (results not shown). Since siRNA must remain intact to interact with the RISC complex, it is likely

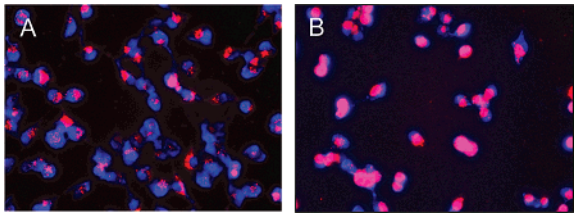
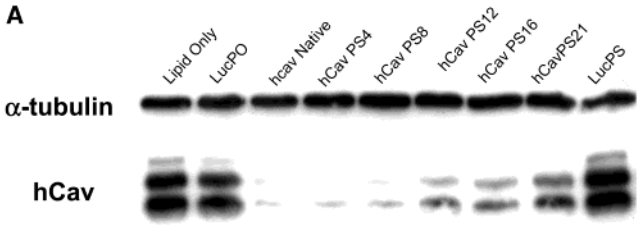


FIGURE 3: Microscopy of uptake of (A) fluorophore-labeled PO duplex RNA and (B) PS duplex RNA. Both PO and PS duplexes were labeled with Cy-3 (pink), and nuclei were labeled with Hoeschst 33258 (blue). 100 nM concentrations of the duplexes were introduced into HeLa cells using OligofectAMINE.



ID	Sequences 5′→3′Sense (+) 3′←5′Antisense (-)	T_m °C
hCav native	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	78
hCav PS4	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	73
hCav PS8	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	69
hCav PS12	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	65
hCav PS16	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	58
hCav PS21	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	68

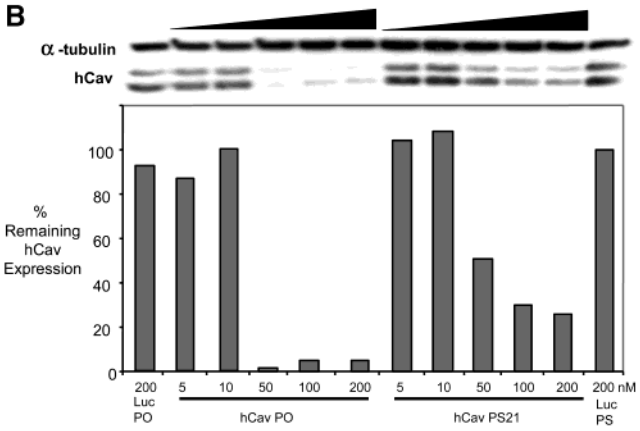


FIGURE 4: (A) Western blot analysis of the inhibition of expression of human caveolin by 200 nM duplex RNA with varying PS substitution. Underlined nucleotides have PS linkages. As negative controls, duplex PO (lucPO) and PS (lucPS) RNAs targeted against firefly luciferase were also introduced into cells. (B) Western analysis comparing inhibition of hCav expression by different concentrations of fully phosphodiester (hCav native) and fully phosphorothioate substituted (hCav PS21) duplex RNA. Luc-PO, a duplex phosphodiester RNA complementary to luciferase. Luc-PS, a duplex RNA complementary to luciferase completely substituted with phosphorothioate linkages. Luc-PO, a duplex RNA complementary to luciferase completely substituted with phosphodiester linkages. Luc-PS, a duplex RNA complementary to luciferase completely substituted with phosphorothioate linkages.

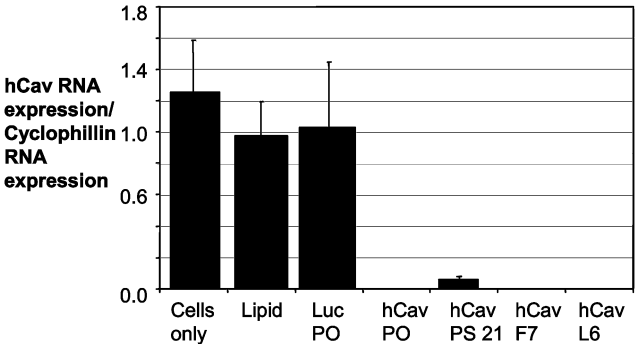


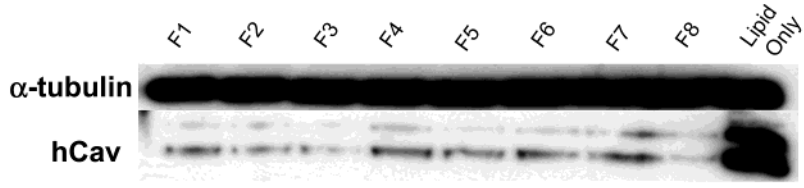
FIGURE 5: Results of RT-PCR measurement of levels of hCav RNA from HeLa cells alone, cells treated with lipid, and cells treated with various RNA duplexes. Results were normalized relative to levels of cyclophilin RNA. The identities of RNA duplexes hCav PO and hCav PS 21 are described in Figure 3A. The identities of 2′-deoxy-2′-fluoro RNA duplex F7 and LNA-RNA duplex L6 are described in Figures 6 and 7, respectively.

that the extreme stability of PO-RNA duplexes makes an important contribution to the robust nature of mammalian RNAi. It is likely that further stability in a cell culture is gained by complexation with lipid, and that once inside cells, the amount of nuclease activity is relatively small.

We also examined the stability in serum of RNAs containing varying numbers of PS substitutions. We had expected that PS substitutions would stabilize RNA because introduction of PS linkages greatly enhances DNA stability (*16*, *17*) and has been reported to stabilize ribozymes when exposed to fetal calf serum (*33*). In fact, we observed that single PS-RNA strands that were substituted with either 12 or 21 (complete) PS linkages were readily degraded (Figure 2A). One explanation for the instability of our single-stranded PS-RNAs as compared to the PS-ribozymes tested previously is that the ribozymes fold into strong tertiary structures that may amplify the stability conferred by the PS linkages. Duplex PS-RNAs containing 12 or 21 PS substitutions per strand were stable during extended incubations as long as 72 h, but stability was no higher than that of PO-RNA duplexes (Figure 2A,B). Similar results were also obtained with PS-RNA duplexes targeted to firefly luciferase (results not shown).

Thermal Stability of Native and Chemically Modified siRNA. The extreme stability of duplex RNA in serum and the extreme instability of single-stranded RNA emphasize the importance of duplex formation. Depending on their sequence, some siRNA duplexes may have melting temperatures that cause them to be relatively unstable at 37 °C. For example, siRNAs that have a high percentage of A/U-basepairs would be expected to have a lower thermal stability than those that have more G/C-basepairs. Low thermal stability might cause these duplexes to dissociate, increasing the likelihood that they will be degraded and rendering them ineffective for RNAi. Therefore, thermal stability will be an important variable to evaluate when considering the value of chemical modifications to siRNA.

Introduction of PS linkages into RNA reduced melting temperature (T_m) values relative to the analogous duplex containing phosphodiester linkages (Figure 4A). The introduction of 2′-deoxy-2′-fluorouridine nucleotides did not significantly increase T_m values (Figure 6), although we note that no duplex possessed more than two base pairs with 2′-



ID	Sequences 5′→3′Sense (+) 3′←5′Antisense (-)	T_m °C
F1	agacgagcugagcgagaagtt **F**tucugcucgacucgcucuuc	79
F2	agacgagcugagcgagaagtt ttucugcucgacucgcucu**F**c	80
F3	agacgagcugagcgagaagtt tt**F**cugcucgacucgcucu**F**c	80
F4	agacgagcugagcgagaagt**F** ttucugcucgacucgcucuuc	79.5
F5	agacgagc**F**gagcgagaagtt ttucugcucgacucgcucuuc	79
F6	agacgagc**F**gagcgagaagt**F** ttucugcucgacucgcucuuc	79
F7	agacgagcugagcgagaagt**F** **F**tucugcucgacucgcucuuc	79.5
F8	agacgagc**F**gagcgagaagt**F** tt**F**cugcucgacucgcucu**F**c	80

FIGURE 6: Western blot analysis of the inhibition of expression of hCav by 100 nM duplex RNA with varying 2′-deoxy-2-fluorouridine substitutions. F = 2′-deoxy-2-fluorouridine.

deoxy-2′-fluoruridine nucleotides and that it is it is possible that additional 2′-deoxy-2′-fluorouridine substitutions would have increased thermal stability significantly. By contrast, introduction of four to eight LNA nucleotides substantially increased T_m values (Figure 7).

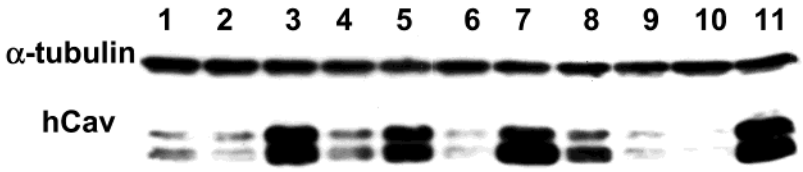
Introduction of siRNAs into HeLa Cells. We added modified siRNAs to HeLa cells in complex with cationic lipid. Confocal microscopy indicated that the fluorophore-labeled PO and PS oligomers entered cells. The localization of fluorophore-labeled PO and PS duplexes appeared to indicate that nuclear uptake of the PS duplex was greater than nuclear uptake of the PO duplex (Figure 3), a result consistent with earlier observations that phosphorothioate DNA/lipid complexes exhibit enhanced nuclear localization (*34*). Fluorescent assisted cell sorting (FACS) and analysis revealed that greater than 80% of cells were successfully transfected with either PS or PO siRNA (results not shown).

Inhibition of hCav Expression by Duplex RNA with Varying PS Substitution. We introduced duplexes with 8−42 PS substitutions per duplex and monitored the inhibition of hCav expression. All of the siRNA duplexes (hCav-PS4 (±), hCav-PS8 (±), hCav-PS12 (±), hCav-PS16 (±), and hCav-PS21 (±) inhibited gene expression (Figure 4A,B). We observe less inhibition by fully PS-modified duplex hCav-PS21 (±). Zeng and Cullen have reported that RISC activity is restricted to the cytoplasm (*35*), and the reduced activity of hCav-PS21 (±) may be related to the greater accumulation in the nucleus observed by microscopy (Figure 3). The PO-RNA duplex reproducibly showed a sharp decrease in inhibition between 50 and 10 nM, while the transition for hCav-PS21(±) was more gradual (Figure 4B). We also measured levels of hCav RNA in cells treated with PO and PS RNA and observed that they were reduced (Figure 5), as would be expected if the RNAs were acting through RNAi. These results demonstrate that RNAi in mammalian cells tolerates substantial substitution with PS linkages.

Our observation that hCav-PS21 can inhibit gene expression without substantial toxicity contrasts with a recent report describing extensive cell death upon introduction of extensively modified duplex PS-RNA into HeLa cells (*29*). We cannot definitively explain this discrepancy but note that oligomers with phosphorothioate modification can be relatively difficult to purify and that impurities that remain after synthesis can increase toxicity upon transfection into cultured cells. The precautions taken in our experiments are described in the Materials and Methods.

Treatment of cells with oligonucleotides that contain PS linkages can cause misleading phenotypes, and it is essential that control experiments be performed to confirm a specific mechanism (*36*). To achieve this confirmation, we performed a series of control experiments that suggest that the observed reduction of hCav expression was due to sequence-specific RNAi. Introduction of either the (+) or the (−) RNA strands alone had no effect on hCav expression regardless of phosphorothioate substitution (results not shown), consistent with the expectation that these single strands would not be stable in serum (Figure 2A). Expression of α-tubulin was not reduced by any of the duplexes, consistent with selective inhibition of gene expression. Finally, RNA duplexes complementary to luciferase did not inhibit caveolin expression (Figure 4A,B), suggesting that complementarity to hCav was necessary for the observed inhibition of hCav.

Inhibition of hCav Expression by Duplex RNAs Substituted with 2′-Deoxy-2′-fluorouridine and 2′-O-Methyl Nucleotides.



ID	Sequences 5′-->3′ 3′<--5′	T_m °C
Unmodified RNA	agacgagcugagcgagaagtt ttucugcucgacucgcucuuc	78
L1	agacgagcugagcgagaagt**T** **T**tucugcucgacucgcucuuc	77
L2	**A**gacgagcugagcgagaagtt ttucugcucgacucgcucuu**C**	76
L3	**A**gacgag**C**ugag**C**gagaagt**T** **T**tucug**C**ucgacucgcu**C**uu**C**	>93
L4	**A**gacgagcugagcgagaagt**T** **T**tucugcucgacucgcucuu**C**	84
L5	agacgag**C**ugag**C**gagaagtt ttucug**C**ucgacucgcu**C**uuc	>93
L6	aga**C**gagcugag**C**gagaagtt ttu**C**ugcucgacucgcu**C**uuc	>93
L7	agac**GA**gcugagcgag**AA**gtt ttu**C**u**G**cucgacucg**C**u**C**uFc	93

FIGURE 7: Western analysis of inhibition of hCav expression by 200 nM RNA duplexes L1−L7 containing LNA nucleotides. Lane 1, RNA-LNA duplex L1; lane 2, RNA-LNA duplex L2; lane 3, RNA-LNA duplex L3; lane 4, RNA-LNA duplex 4; lane 5, LNA-RNA duplex 5; lane 6, LNA-RNA duplex 6: lane 7, LNA-RNA duplex 7; lane 8, LNA-RNA 7 (+) strand hybridized to native RNA (−) strand; lane 9, LNA-RNA 7 (−) strand hybridized to native RNA (−) strand; lane 10, duplex native RNA; and lane 11, lipid only, no nucleic acid added. LNA substitutions are capitalized and in bold.

2′-Deoxy-2′-fluorouridine and 2′-*O*-Me nucleotides are used for optimizing classical antisense oligonucleotides (*19*−*21*), and we sought to determine whether these modifications were compatible with RNAi in human cells. 2′-Deoxy-2-fluorouridine substitutions were introduced at the 3′-termini of the RNA strands or internally. In all cases, the resulting duplexes (F1−F8) caused efficient inhibition of hCav expression upon introduction into cells (Figure 6), demonstrating that RNAi tolerates at least partial substitution with 2′-deoxy-2′-fluoro nucleotides, providing another option for optimizing RNAi in mammalian cells. We also measured levels of hCav RNA in cells treated with 2′-deoxy 2′-fluoro substituted RNA and observed that they were reduced (Figure 5).

Dose response data indicated that the potency of inhibition by duplexes with terminal or internal 2′-deoxy-2′-fluorouridine substitutions was similar to native RNA (results not shown). Single strands containing 2′-deoxy-2′-fluorouridine substitutions did not inhibit gene expression when introduced into cells (results not shown). Consistent with reports by Tuschl of studies of RNAi in *Drosophila* cell extract (*26*), we failed to observe inhibition of gene expression upon transfection of fully substituted 2′-*O*-meRNA in one or both strands of the duplex (results not shown).

Inhibition of hCav Expression by Duplex RNAs Substituted with LNA Nucleotides. The introduction of LNA nucleotides increases the thermal stability of DNA oligonucleotides by as much as 10 °C per substitution (*22*−*24*). Therefore, we hypothesized that the introduction of LNA nucleotides into RNA might be used to increase T_m values for siRNA duplexes while retaining high activity for inhibition of gene expression.

To test this hypothesis, we synthesized a series of LNA-RNA chimera containing LNA nucleotides at either the 3′- or the 5′-termini or internally for their ability to inhibit gene expression. Belief that the proteins that make up the RNA induced silencing complex (RISC) would recognize LNA-RNA chimera was encouraged by findings that RNAse H is able to recognize and cleave hybrids between DNA-LNA chimeras and RNA (*37*) and that DNA-LNA chimeras can be effective antisense agents for blocking gene expression inside cells at concentrations as low as 5−20 nM (*38*).

We observed that introduction of LNA nucleotides increased T_m values for hybridization of LNA-RNA chimera, with just four LNA substitutions within the duplex increasing T_m values by as much as 14 °C (Figure 7). LNA-RNA duplexes L1, L2, and L4 that contain LNA nucleotides at 3′-, 5′-, or both 3′- and 5′-termini were able to block hCav expression upon introduction into cells (Figure 7, lanes 1, 2, and 4). These results indicate that the RISC complex can recognize the termini of these oligomers despite the substantial geometric constraint imposed by the 2′−4′ methylene bridge and are similar to findings by Taira and co-workers using terminal nucleotides with a 2′−4′ ethylene bridge (*39*).

LNA-RNA duplex L6 that contains internal LNA substitutions and has a T_m of greater than 95 °C was also able to block hCav expression (Figure 7, lane 6). This result led us

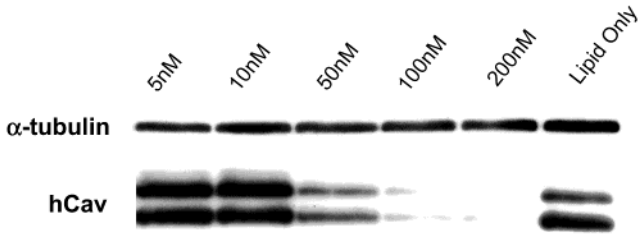


FIGURE 8: Dose response Western analysis of inhibition of hCav expression by chimera LNA-RNA L6 containing LNA bases on both strands (5′-aga**C**gagcugag**C**gagaagtt-3 and 3′-ttu**C**ugcucgacucgcu**C**uuc-5′) (LNA substitutions are capitalized and in bold).

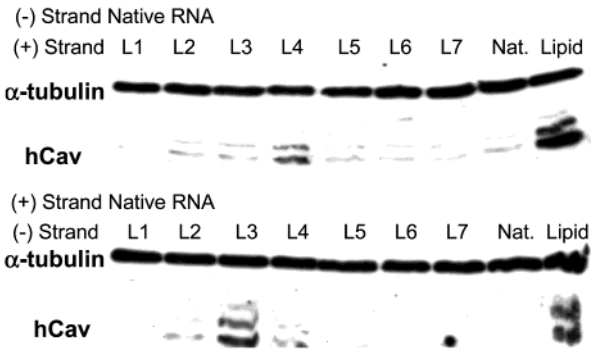


FIGURE 9: Western analysis of inhibition of hCav expression by (+) or (−) RNA strands L1−L7 containing LNA nucleotides in complex with the complementary (−) or (+) strands of native unmodified RNA. Nat.: native, unmodified RNA (hCav-PO). Lipid: lipid added to cells without any oligomer. All duplexes were present at 200 nM.

to examine inhibition of hCav expression as a function of the concentration of LNA-RNA L6 and observed that it was as efficient as the parent siRNA duplex (Figure 8). Efficient inhibition by L6 demonstrates that T_m values for RNA duplexes can be dramatically increased without affecting the function of the RISC complex. This is consistent with evidence that RNAi functions by an ATP dependent mechanism (*40*, *41*) in which the energy released by ATP hydrolysis drives separation of the siRNA strands. By contrast, LNA-RNA duplexes L3, L5, and L7 that contained internal LNA substitutions were ineffective and did not block hCav expression (Figure 7, lanes 3, 5, and 7). We also measured levels of hCav RNA in cells treated with LNA-RNA duplexes and observed RNA levels that parallel levels of hCav protein (Figure 5, results of treatment with L6 shown).

The reason for the different efficiencies of L6 relative to L3, L5, and L7 is not obvious because they possess similar T_m values and have LNA substitutions at similar positions. However, we note that inactive LNA-RNAs L3 and L5 have two LNA nucleotides within the central seven base region of the antisense strand, while active LNA-RNA L6 has only one LNA base within this region. LNA-RNA L7 may be inactive because it has four substitutions in each strand, twice as many as active LNA 6.

To more closely investigate the effects of LNA substitution of RNAi, we evaluated duplexes consisting of one native RNA strand and one LNA-RNA strand. When the (+) or the (−) strands of inactive LNA-RNA duplex L7 are paired with native RNA strands, inhibition of gene expression is observed (Figure 9; lanes 8 and 9, Figure 6) indicating that the placement of LNA nucleotides on the individual single

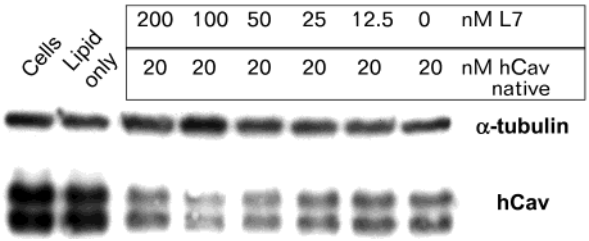


FIGURE 10: Effect of adding increasing amounts of inactive LNA-RNA chimera L7 on inhibition of hCav expression by 20 nM non LNA-containing duplex RNA (PO-siRNA, hCav native described in Figure 3A).

strands was not responsible for the lack of inhibition by the duplex. This experiment led us to extend this analysis of duplexes consisting of native unmodified RNA hybridizing to other LNA-RNA chimera. We observed significant inhibition of gene expression for all duplexes except the pairings of native sense strand RNA with the antisense L3 strand (Figure 9). T_m values were significantly increased for native RNA in combination with (+) or (−) strands of duplex L3 (86 and 90 °C, respectively) and for native RA with (+) or (−) strands of L7 (84 and >93 °C, respectively). These data demonstrate that LNA substitution of just one strand is adequate to generate increased T_m values.

These data suggest that LNA substitutions are well-tolerated and can lead to large increases in T_m values. However, to maximize the likelihood that potent inhibition of gene expression will be maintained, LNA substitutions should be kept to a minimum and should not infringe on the central region of the RNA. It is not difficult to meet these criteria since only modest numbers of LNA substitutions are necessary to significantly increase T_m. Combining the thermal stability contributed by LNA substitutions with the possible pharmacokinetic advantages afforded by phosphorothioate linkages may provide a useful strategy for optimizing RNAi efficacy in animals.

Inactive LNA-RNA Duplexes Do Not Inhibit RNAi. One explanation for the inactivity of LNA-RNA duplexes L3, L5, and L7 is that they might bind to the RISC complex but not be processed. If this explanation were true, these complexes might act as competitive inhibitors of RNAi by blocking processing of active RNA duplexes. We find, however, that introduction of increasing concentrations of duplexes L3, L5, or L7 into cells has no effect on inhibition of caveolin by the analogous native RNA duplex that lacks LNA nucleotides (results for L7 are shown in Figure 10). L3, L5, and L7 may be unable to bind to the RISC complex, or binding may be insufficient to block binding of native RNA.

Classical Antisense Approaches and siRNA. Classical antisense approaches to the inhibition of gene expression have been developed that use chemically modified single-stranded DNA or RNA and have been used with success for target validation and drug development (*1*−*4*). Two features of siRNA differ from these classical approaches: (i) duplex RNA is used and (ii) the mechanism of inhibition involves the RISC, which promotes recognition and cleavage of the mRNA target. These are important differences. Nevertheless, they should not conceal the fundamental similarities of the two approaches, and Vickers and co-workers report that when classical antisense is optimized it can be as effective as RNAi (*42*). Both RNAi and classical antisense strategies for inhibition of gene expression require recognition of mRNA,

both can employ synthetic nucleic acids, and both require well-controlled experiments to demonstrate specificity for a specific target.

Chemical Modifications and Improved siRNA. By analogy with classical antisense, areas for improvement of siRNA might include enhanced nuclease resistance, higher potency, higher specificity, greater thermal stability, more efficient cellular uptake, better biodistribution, and better pharmacokinetics. Perhaps the single most important modification of traditional antisense oligonucleotides is phosphorothioate linkages. While PS linkages are an efficient means to stabilize DNA and have been reported to stabilize ribozymes (*33*), they do not noticeably stabilize the RNA single strands used in these studies (Figure 2), and we observe that duplex RNA is already remarkably stable even in the absence of PS linkages.

If PS substitutions are not needed to stabilize siRNA, is there a role for them in optimization of RNAi? PS substitutions are known to improve the pharmacokinetics of traditional antisense oligonucleotides by increasing binding to serum proteins. This association reduces clearance rates and improves the in vivo half-life in circulation (*18*). For single-stranded oligonucleotides, as few as 13 PS substitutions are needed to confer useful pharmacokinetic properties (*18*). We find that duplex RNAs containing between eight and 42 PS linkages retain the ability to inhibit gene expression (Figure 4A), so there is wide latitude for using PS linkages to optimize pharmacokinetic parameters.

Another goal for use of chemical modifications in traditional antisense strategies is to increase the thermal stability of hybridization. Thermal stability might become limiting for RNA duplexes that are relatively AT-rich or if other substitutions, such as PS linkages, are introduced that tend to reduce T_m values. Our results indicate that the introduction of LNA nucleotides can substantially stabilize RNA duplexes without loss of the ability to inhibit gene expression. Inhibition of gene expression by LNA-substituted oligomers that have high (>93 °C) T_m values also provides valuable mechanistic information, indicating that the RISC complex is able to dissociate RNA duplexes that possess exceptionally high thermal stability.

ACKNOWLEDGMENT

We thank the referees of this manuscript for their helpful comments.

REFERENCES

1. Koller, E., Gaarde, W. A., and Monia, B. P. (2000) *Trends Pharm. Sci. 21*, 142−148.
2. Jansen, B., and Zangemeister-Wittke, U. (2002) *Lancet Oncol. 3*, 672−683.
3. Opalinska, J. B., and Gewirtz, A. M. (2002) *Nature Rev. Drug Discov. 1*, 503−514.
4. Braasch, D. A., and Corey, D. R. (2002) *Biochemistry 41*, 4503−4510.
5. Elbashir, S. M., Harboth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature 411*, 494−498.
6. Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) *Methods 26*, 199−213.
7. Tuschl, T., and Borkhardt, A. (2002) *Mol. Interventions 2*, 158−167.
8. Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000) *Nature 404*, 293−296.
9. Hammond, S. M., Boetcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. (2001) *Science 293*, 1146−1150.
10. Zamore, P. D. (2001) *Nat. Struct. Biol. 8*, 746−750.
11. Wianny, F., and Zernicka-Goetz, M. (2000) *Nat. Cell Biol. 2*, 70−75
12. Xia, H. B., Mao, Q., Paulson, H. L., and Davidson, B. L. (2002) *Nat. Biotech. 20*, 1006−1010
13. McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. (2002) *Nature 418*, 38−39.
14. Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., and Lieberman, J. (2003) *Nat. Med. 9*, 347−351.
15. Freier, S. M., and Altmann, K. H. (1997) *Nucl. Acids Res. 25*, 4429−4443.
16. Eckstein, F. (2000) *Antisense Nucl. Acid Drug Dev. 10*, 117−121.
17. Eckstein, F. (2002) *Biochimie 84*, 841−848.
18. Geary, R. S., Yu, R. Z., and Levin, A. A. (2001) *Curr. Opin. Invest. New Drugs 2*, 562−573.
19. Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., and Freier, S. M. (1993) *J. Biol. Chem. 268*, 14514−14522.
20. Kawasaki, A. M., Casper, M. D., Freier, S. M., Lesnik, E. A., Zounes, M. C., Cummins, L. L., Gonzalez, C., and Cook, P. D. (1993) *J. Med. Chem. 36*, 831−841.
21. Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., and Freier, S. M. (1993) *J. Biol. Chem. 268*, 14514−14522.
22. Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K, Kumar, R., Meldgaard, M., Olsen, C. E., and Wengel, J. (1998) *Tetrahedon 54*, 3607−3630.
23. Obika, S., Nanbu, D., Hari, Y., Andoh, J., Morio, K., Doi, T., and Imanishi, T. (1998) *Tetrahedron Lett. 39*, 5401−5404.
24. Braasch, D. A., and Corey, D. R. (2001) *Chem. Biol. 8*, 1−7.
25. Parrish, S., Fleenor, J., Xu, S., Mello, C., and Fire, A. (2001) *Mol. Cell 6*, 1077−1087.
26. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001) *EMBO J. 20*, 6877−6888.
27. Schwartz, D. S., Hutvagner, G., Haley, B., and Zamore, P. D (2002) *Mol. Cell 10*, 537−548.
28. Rana, T. M., and Chiu, Y.-L. (2002) *Mol. Cell 10*, 549−561.
29. Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003) *Nucl. Acids Res. 31*, 589−595.
30. Smart, E. J., Graf, G. A., McNiven, M. A., Sessa, W. C., Engelman, J. A., Scherer, P. E., Okamoto, T., and Lisanti, M. P. (1999) *Mol. Cell. Biol. 19*, 7298−7304.
31. Liu, P., Rudick, M., and Anderson, R. G. W. (2002) *J. Biol. Chem. 277*, 41295−41298.
32. Moskalenko, S., Henry, D. O., Rosse, C., Mirey, G., Carmonis, J. H., and White, M. A. (2001) *Nature Cell Biol. 4*, 66−72.
33. Heidenreich, O., Benseler, F., Fahrenholtz, A., and Eckstein, F. (1994) *J. Biol. Chem. 269*, 2131−2138.
34. Marcusson, E. G., Bhat, B., Manoharan, M., Bennet, C. F., and Dean, N. M. *Nucl. Acids Res. 26*, 2016−2023.
35. Zeng, Y., and Cullen, B. (2002) *RNA 8*, 855−860.
36. Stein, C. A. (1999) *Biochim. Biophys. Acta 1489*, 45−52.
37. Kurreck, J., Wyszko, E., Gillen, C., and Erdmann, V. A. (2002) *Nucl. Acids Res. 30*, 1911−1918.
38. Braasch, D. B., Liu, Y., and Corey, D. R. (2002) *Nucl. Acids Res. 30*, 5160−5167.
39. Homada, M., Ohtsuka, T., Kawaida, R., Koizuma, M., Morita, K., Furukawa, H., Imanishi, T., Miyagishi, M., and Taira, K. (2002) *Antisense Nucl. Acid Drug Dev. 12*, 301−309.
40. Zamore, P. D., Tuschl, T., Sharp, P. A, and Bartel, D. P. (2000) *Cell 101*, 25−33.
41. Nykanen, A., Haley, B., and Zamore, P. D. (2001) *Cell 107*, 309−321.
42. Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2002) *J. Biol. Chem. 278*, 7108−7118.

BI0343774