Improving RNA Interference in Mammalian Cells by 4′-Thio-Modified Small Interfering RNA (siRNA): Effect on siRNA Activity and Nuclease Stability When Used in Combination with 2′-*O*-Alkyl Modifications

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A systematic structure−activity relationship study of 4′-thioribose containing small interfering RNAs (siRNAs) has led to the identification of highly potent and stable antisense constructs. To enable this optimization effort for both in vitro and in vivo applications, we have significantly improved the yields of 4′-thioribonucleosides by using a chirally pure (*R*)-sulfoxide precursor. siRNA duplexes containing strategically placed regions of 4′-thio-RNA were synthesized and evaluated for RNA interference activity and plasma stability. Stretches of 4′-thio-RNA were well tolerated in both the antisense and sense strands. However, optimization of both the number and placement of 4′-thioribonucleosides was necessary for maximal potency. These optimized siRNAs were generally equipotent or superior to native siRNAs and exhibited increased thermal and plasma stability. Furthermore, significant improvements in siRNA activity and plasma stability were achieved by judicious combination of 4′-thioribose with 2′-*O*-methyl and 2′-*O*-methoxyethyl modifications. These optimized 4′-thio-siRNAs may be valuable for developing stable siRNAs for therapeutic applications.

Introduction

Small interfering RNAs (siRNAs) are short 19−21-nucleotide RNA duplexes that regulate gene expression via the RNA interference (RNAi) pathway.[1−7] Over the past few years several groups have reported the use of chemically synthesized siRNAs to control gene expression in cultured mammalian cells.[1,2,8] siRNAs introduced into cells using cationic lipid transfection agents have shown significant reduction in target mRNA levels.[2,9,10] Recent reports also provide preliminary evidence of in vivo siRNA activity in mouse models using high-pressure tail vein injections.[11,12] Although detectable levels of siRNAs were found in liver tissue in these mice, this method of administration is unsuitable for clinical applications. Sorensen et. al. have reported gene silencing by a cationic liposome based intravenous co-injection of siRNAs and a reporter plasmid.[13] More recently, Soutschek et. al. have demonstrated endogenous gene silencing by a systemically administered siRNA−cholesterol conjugate.[14] These reports offer preliminary evidence of the therapeutic potential of siRNAs and warrant further improvements in siRNA design with a view toward improving its potency and pharmacokinetic properties.

The biodistribution of phosphodiester and phosphorothioate siRNAs in mice has been reported to be similar to that for antisense oligonucleotides (ASOs) following intravenous or intraperitoneal injection.[15] Resistance to degradation by nucleases is one of the key factors which determine the bioavailability, in vivo potency, and efficacy of ASOs.[10,16−18] Furthermore, since degradation in both tissues and plasma is observed via 3′-exo-, 5′-exo-, and endonucleases, stabilizing all parts of the ASO drug will likely be required to achieve the maximum therapeutic benefit. Since there is growing evidence that unmodified siRNAs are rapidly degraded in serum,[15,19−21] identifying highly stabilized siRNA constructs is crucial to the development of siRNA therapeutics.

Chemical modifications have long been used to effectively improve the serum stability of ASOs,[16−18,22−24] and several groups have sought to develop a similar strategy for siRNAs.[6,15,19−21,25−29] Czauderna et. al. have reported increased duration of siRNA activity in mammalian cells with increasing nuclease stability of siRNAs.[26] Although several investigators have incorporated nuclease-resistant 2′-*O*-alkyl modifications and locked nucleic acids into siRNAs, these modifications have generally led to reduced potency when compared with parent unmodified siRNAs.[6,15,19−21,25−29] However, thorough optimization of the placement of modifications in each strand can lead to derivatives with both improved potency and improved stability, as exemplified by our recent report on fully modified siRNA duplexes having improved activity.[30]

We have also recently reported on the positional effects of well-known RNA-stabilizing 2′-ribose modifications, namely, 2′-*O*-methyl (2′-*O*-Me), 2′-α-fluoro (2′-F), and 2′-*O*-methoxyethyl (2′-*O*-MOE), on siRNA activity.[31] These studies clearly demonstrated that 2′-substitution at the 5′-end of the antisense strand tended to reduce potency, and that the reduction correlated with the steric bulk of the substituent. The exceptions to this general rule are the alternating 2′-*O*-Me motifs with either RNA[26] or 2′-F[30] which improve both stability and potency in certain cases. However, these observations have not been generalized to broad tolerance for other stabilizing modifications at the 5′-end of the antisense strand. Since nuclease resistance generally correlates positively with both the number and bulk of 2′-substituents, additional methods to stabilize the 5′-end of antisense strand siRNAs are needed.

Another way to enhance nuclease resistance and plasma−protein binding of oligonucleotides is to introduce sulfur into the molecule by using phosphorothioate oligonucleotides.[16,18,22] These have been used extensively in antisense therapeutics to generate serum-stable oligonucleotides with increased plasma−protein binding and plasma residence time (as compared to those of phosphodiester oligonucleotides). However, a similar strategy

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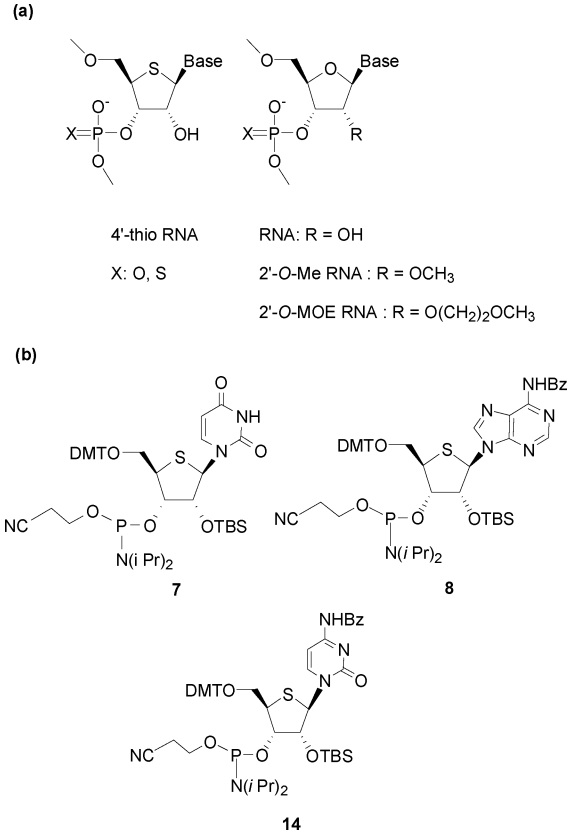


Figure 1. (a) 4′-Thio-RNA and 2′-*O*-modified RNA. Base = Ade, Gua, Cyt, or Ura. (b) 4′-Thioribonucleoside 3′-phosphoramidites used in 4′-thio-RNA synthesis.

when applied to siRNAs resulted in reduced activity as compared to that of native phosphodiester siRNAs.[31] Incorporation of a thioether into the 2′-substitutent of single-stranded oligonucleotides has resulted in improved binding to human serum albumin.[32] As the binding of oligonucleotides to plasma proteins has been positively correlated to in vivo tissue distribution,[18] the introduction of sulfur is expected to improve the pharmacokinetic properties of siRNA by both increasing the stability toward degradation and improving the distribution to tissues. However, this 2′-modified sulfur-containing modification is not ideal for siRNA, as it is expected to decrease activity when incorporated into the antisense strand due to its large steric bulk as is evident from our previous results with 2′-*O*-MOE modifications.[31]

In contrast, a sulfur-containing modification seemingly ideal for use in siRNAs is 4′-thio-RNA, synthesized from 4′-thioribonucleosides that have a sulfur atom at the 4-position of the thiofuranose ring (Figure 1a). Although 4′-thioribonucleosides share the C3′-endo sugar pucker and anti conformation with ribonucleosides, they exhibit some key structural and electronic differences that make them attractive for siRNA applications. Sulfur is larger and less electronegative than oxygen, and as such the C−S bond length is longer (1.82 Å), the S−C−N anomeric effect is weaker, and the endocyclic C−S−C angle is more acute in 4′-thioribonucleosides.[33−35] The lower electronegativity of the sulfur in 4′-thioribonucleosides also causes a perturbation in the C−N glycosidic bond, altering its chemical reactivity when compared to the C−N bond in ribonucleosides.[36] These properties presumably contribute to their resistance to nucleoside phosphorylases and phosphatases. However, the electronic environment of the nucleobases is not significantly perturbed, and neither are their hydrogen-bonding properties. 4′-Thioribonucleosides are also poor substrates for bovine liver adenosine kinase and do not show substrate inhibition of the enzyme.[37] Thus, kinase resistance, metabolic stability, and H-bonding abilities make 4′-thioribonucleosides interesting candidates for incorporation into siRNA duplexes to enhance siRNA serum stability and possibly protein binding properties.

The synthesis and characterization of 4′-thio-RNA was first reported by Imbach et. al [38−41] and more recently by Hoshika et al.[42−44] They showed that phosphodiester 4′-thio-RNA oligomers were significantly more resistant to cleavage by serum nucleases than unmodified phosphodiester RNA. Furthermore, 4′-thio-RNA hybridizes with complementary RNA sequences to form stable duplexes with standard A-form helical geometry. These properties make 4′-thio-RNA a structurally similar, yet nuclease-resistant analogue of wild-type RNA and hence attractive for siRNA applications.

In a recent report by Hoshika et. al. on the siRNA activity of 4′-thio-RNA in cultured NIH/3T3 cells against photinus luciferase,[43] it was shown that 4′-thio-RNA is tolerated predominantly in the sense strand, and that incorporation into the antisense strand in large numbers leads to greater than 10-fold reductions in potency. The best construct tested consisted of modifications on the sense strand alone, and showed similar potency. In our preliminary communications we independently reported utility of 4′-thioribose-modified siRNA in down-regulating the mRNA of an endogenous gene (PTEN).[45,46] However, the siRNAs with a minimum number of 4′-thioribose-modified residues resulted in good activity but a small benefit in stability toward nucleases. Because of our previous success in optimizing the placement of chemical modifications in siRNAs,[30,31] we set out to carry out a detailed analysis of the structure−activity relationship (SAR) of 4′-thio-RNA-containing siRNAs to identify 4′-thioribose-modified motifs that will lead to both improved potency and a significant enhancement in nuclease stability.

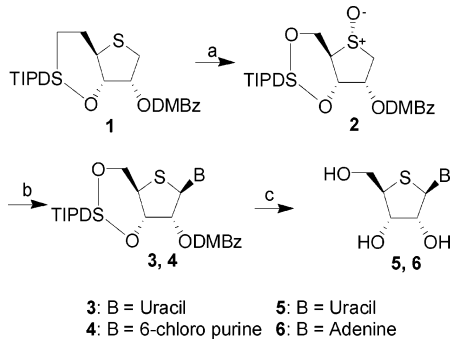
Recent crystallography studies[47,48] showing that the 5′-end of the antisense strand interacts strongly with the RNase H like RISC endonuclease highlight the importance of minimal modification at the 5′-end of the antisense strand. These observations and the known intolerance for bulky substituents at the 5′-end of the antisense strand, combined with the nuclease stability benefit provided by 4′-thio-RNA, suggest that 4′-thio-RNA could be a uniquely suited modification for improving the pharmaceutical properties of siRNAs. Our experimental designs were further based on reports suggesting that the RISC complex is able to differentially select one of the two strands depending on the duplex stability of the first several nucleotides.[49,50] We hypothesized that a similar bias should be possible using the 4′-thioribose modification in combination with bulkier 2′-*O*-Me and especially 2′-*O*-MOE (Figure 1a) on the sense strand in such a way that would lead to preferential loading of the antisense strand.[31] On the basis of these criteria, we have designed, prepared, and evaluated a series of 4′-thio-RNA-containing siRNAs for potency in reducing the target message as well as nuclease stability.

While it remains to be seen with subsequent in vivo pharmacokinetic and pharmacology studies, the incorporation of significant numbers of 4′-thio-RNA residues into the potent and stable constructs identified herein may also contribute to improved tissue distribution required to achieve a robust therapeutic effect in animals.

Results and Discussion

Synthesis of 4′-Thioribose Nucleosides and Phosphoramidites. The synthesis of 4′-thioribonucleosides (Scheme 1) and

Scheme 1[a]



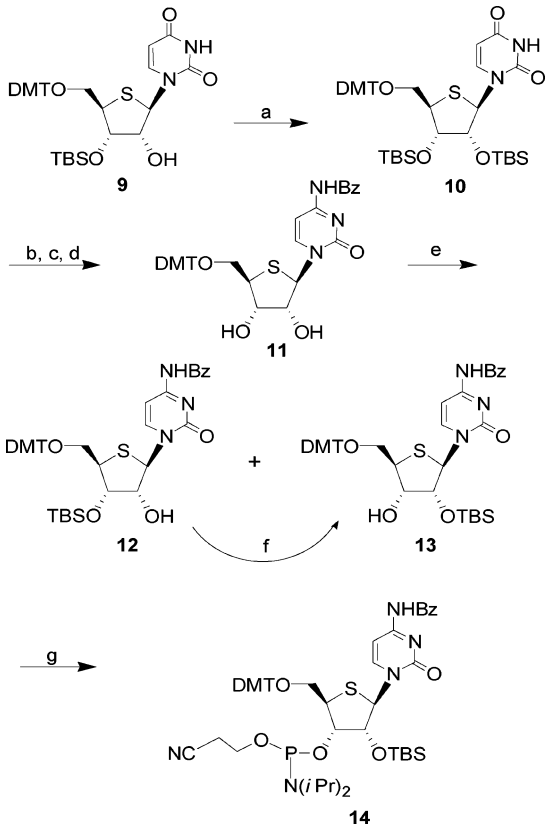
[a] Reagents and conditions: (a) diethyl L-tartrate, titanium(IV) isopropoxide, *tert*-butyl hydroperoxide, CH_2Cl_2, −25 °C; (b) for **3**, trimethylsilyl triflate, triethylamine, 1:1 toluene/CH_2Cl_2, rt; for **4**, trimethylsilyl triflate, triethylamine, 1:1 1,2-dichloroethane/CH_3CN, reflux; (c) (i) TBAF, AcOH, THF; (ii) aqueous ammonia.

their phosphoramidites (Figure 1b) has been described previously.[40,42,43,51,52] However, significantly improved yields were obtained by making a key improvement in the synthetic methodology, which is detailed below.

Arguably, the key step in the synthetic route is the oxidative conversion of **1** to **2** (Scheme 1). The reported procedures for converting **1** to **2** gave acceptable yields (60−80%) as a mixture of (*R*)- and (*S*)-sulfoxides (*R*:*S* = 2.7:1 with mCPBA, 16:1 by ozonolysis). Unfortunately, only the *R* isomer undergoes productive glycosylation under Pummerer conditions while the *S* isomer undergoes elimination side reactions.[51] Although ozonolysis gave an improved ratio of *R* to *S* isomers, in our hands this procedure gave consistently low yields (60%) and was exceedingly difficult to scale up. We therefore investigated alternate oxidation methods that could give us chirally pure **(***R***)-2** and which could be rapidly and consistently scaled up. By using a Di Furia−Modena oxidation[53−56] (a mixture of *tert*-butyl hydroperoxide, diethyl L-tartrate, and titanium(IV) isopropoxide), we were able to control the stereochemistry of the oxidation product. Only the desired (*R*)-sulfoxide was obtained in 90% isolated yield with no detectable *S* isomer (as indicated by ^{1}H NMR). Importantly, this procedure has been scaled up to a 500 g scale with 92% isolated yields and without chromatographic purification. When we used chirally pure **(***R***)-2** as the sugar donor, the Pummerer thioglycosylation[51] gave yields of up to 97% for pyrimidines and up to 70% for purines. Thus, stereoselective sulfur oxidation directly results in increased overall synthetic yields and represents an important synthetic improvement.

Compounds **5** and **6** (Scheme 1) were converted into their corresponding phosphoramidites (Figure 1b, **7** and **8**) via standard procedures.[52,57] The synthesis of 4′-thio-N^4-benzoylcytidine 3′-phosphoramidite was accomplished as described in Scheme 2. Compound **9**, a byproduct obtained[40] during the synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-4′-thiouridine, was silylated at the 3′-position with *tert*-butyldimethylsilyl chloride [(TBS)Cl], imidazole, and pyridine at room temperature to yield **10**. Compound **10** was converted to the triazolide derivative with 1,2,4-triazole, $POCl_3$, and triethylamine.[58] It is important to note that the formation of the triazolide is significantly slower (12 h) in the case of 4′-thioribonucleosides as compared to ribonucleosides (1 h). Subsequent treatment with aqueous ammonia/dioxane (1:1) at ambient temperature for 18 h yielded a cytidine analogue. The exocyclic amino group was protected with a benzoyl group, followed by removal of the silyl group to give **11** (96%). Compound **11** was treated with (TBS)Cl in a mixture of pyridine

Scheme 2[a]

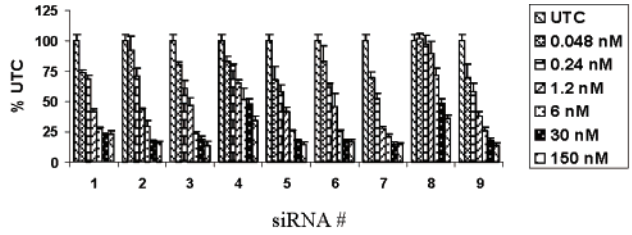


[a] Reagents and conditions: (a) (TBS)Cl, imidazole, 4-(dimethylamino)pyridine, DMF; (b) 1,2,4-triazole, triethylamine, $POCl_3$, CH_3CN; then NH_4OH, 1,4-dioxane; (c) benzoic anhydride, pyridine; (d) triethylamine trihydrofluoride, triethylamine, THF; (e) (TBS)Cl, $AgNO_3$, pyridine, THF; (f) 1% triethylamine in ethanol containing 0.1% imidazole; (g) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, *N*-methylimidazole, DMF.

and THF in the presence of silver nitrate to yield a mixture of 2′-and 3′-silyl derivatives (**12**/**13**). The two regioisomers were separated using flash silica gel column chromatography. The 2′-*O*-TBS derivative **13** was converted to 3′-phosphoramidite **14** according to the reported procedure.[59] Although this chemical transformation has been reported for ribonucleosides,[58] this is the first report of this method being applied to 4′-thioribonucleosides.

Target Gene and Sequence. siRNA duplexes targeting the mature human PTEN messenger RNA (mRNA) were identified by screening a series of siRNA constructs targeting the gene. Unmodified siRNA-**2** (Figure 2) is a 20-base-pair RNA duplex which reduces endogenous PTEN mRNA levels with an $IC_{50} \approx 0.9$ nM when transfected into HeLa cells. siRNA-**12** (Figure 3) is a 19-base-pair duplex which targets the same site on the PTEN messenger RNA with an $IC_{50} \approx 0.4$ nM.

siRNA Design. There is some evidence that different regions of the siRNA play distinct roles in target recognition, cleavage, and product release.[50] Thus, the nature of the chemical modifications as well as their placement within the siRNA may influence RISC loading as well as subsequent processes in the RNAi mechanism.[2,4,6,7,27,28,50] We have recently reported on the positional effects of 2′-F, 2′-*O*-Me, and 2′-*O*-MOE modifications on siRNA-mediated target reduction in mammalian cells.[31] Here we have used a similar strategy to evaluate the positional effect of 4′-thio modifications on siRNA activity. The 4′-thioribose, 2′-*O*-Me, or 2′-*O*-MOE nucleoside residues were systematically introduced into the sense and/or antisense strands, and their

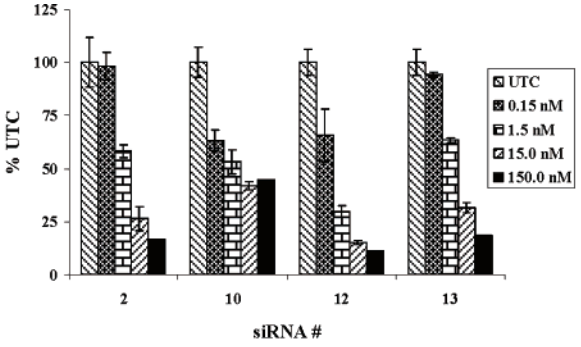


siRNA No.	Sequence				IC_{50}(nM)
siRNA-1	5′ AAGUAAGGACCAGAGACAAA	3′	s		1.6
	3′ UUCAUUCCUGGUCUCUGUUU-P	5′	as		
siRNA-2	5′ AAGUAAGGACCAGAGACAAA	3′	s		0.95
	3′ UUCAUUCCUGGUCUCUGUUU-P	5′	as		
siRNA-3	5′ AAGUAAGGACCAGAGACAAA	3′	s		0.88
	3′ **UUC**AUUCCUGGUCUCUGUUU-P	5′	as		
siRNA-4	5′ AAGUAAGGACCAGAGACAAA	3′	s		14
	3′ UUCAU**UCC**UGGUCUCUGUUU-P	5′	as		
siRNA-5	5′ AAGUAAGGACCAGAGACAAA	3′	s		0.48
	3′ UUCAUU**CCU**GGUCUCUGUUU-P	5′	as		
siRNA-6	5′ AAGUAAGGACCAGAGACAAA	3′	s		1.1
	3′ **UUC**AUUCCUGGUCUCUGUUU-P	5′	as		
siRNA-7	5′ AAGUAAGGACCAGAGACAAA	3′	s		0.24
	3′ **U**UCAUUCCUGGUCUCUGUUU-P	5′	as		
siRNA-8	5′ AAGUAAGGACCAGAGACAAA	3′	s		41.5
	3′ UUCAUUCCUGG**UCU**CUGUUU-P	5′	as		
siRNA-9	5′ AAGUAAGGACCAGAGACAAA	3′	s		0.45
	3′ UUCAUUCCUGGUCUCUG**UUU**-P	5′	as		

Figure 2. Positional effects of 4′-thioribonucleoside residues on siRNA activity. Dose-dependent reduction of the PTEN mRNA level in HeLa cells by siRNA duplexes containing 4′-thioribonucleoside residues in the antisense strand of siRNA duplexes. The 4′-thioribonucleoside residues are indicated in red. The underlined sequence has a phosphorothioate backbone. UTC = untreated control.

effect on siRNA activity was studied as a function of their position within the siRNA duplex. In initial experiments the 5′- end of the modified antisense strand was phosphorylated chemically to generate a 5′-phosphorylated siRNA. This was done to minimize the effect of chemical modification on siRNA phosphorylation by cellular kinases (reported to be essential for siRNA activity).[1] However, we did not observe any difference in siRNA activity when using 5′-phosphorylated or 5′-nonphosphorylated antisense strands, and subsequent studies were carried out without 5′-phosphorylation.

Antisense Strand SAR. Although 4′-thio-RNA is globally similar in structure to unmodified RNA, the substitution of oxygen with sulfur[32,33] may result in localized structural perturbations. The endocyclic oxygen to sulfur substitution may also influence the ability of siRNA duplexes to serve as substrates for enzymes involved in eliciting siRNA activity. These effects are likely to depend on not only the number of modifications but also their position within the siRNA duplex. Hence, the positional effect of 4′-thio modifications on siRNA activity was studied. Optimal positioning of these residues within the antisense strand of the siRNA was studied by walking them



siRNA No.	Sequence				IC_{50}(nM)
siRNA-2	5′ AAGUAAGGACCAGAGACAAA	3′	s		0.95
	3′ UUCAUUCCUGGUCUCUGUUU-P	5′	as		
siRNA-10	5′ AAGUAAGGACCAGAGACAAA	3′	s		5.9
	3′ **UUC**AUUCCUGGUCUC**U**G**UUU**-P	5′	as		
siRNA-12	5 AAGUAAGGACCAGAGACAA	3′	s		0.4
	3′ UUCAUUCCUGGUCUCUGUU	5′	as		
siRNA-13	5′ AAGUAAGGACCAGAGACAA	3′	s		1.2
	3′ **UU**CAUUCCUGGUCUCUG**UU**	5′	as		

Figure 3. Positional effects of 4′-thioribonucleoside residues in the antisense strand on siRNA activity. Dose-dependent reduction of PTEN mRNA in HeLa cells by siRNA gapmer duplexes containing 4′-thioribonucleoside residues at the 3′- and 5′-termini of the antisense strand. The 4′-thioribonucleoside residues are indicated in red. UTC = untreated control.

in the 5′ to 3′ direction on the antisense strand (siRNA-**3** to siRNA-**9**, Figure 2). RNAi activity in reducing PTEN mRNA levels was measured in HeLa cells (Figure 2). As a positive control, siRNA-**1** having a phosphorothioate antisense strand paired with an unmodified sense strand was tested in parallel. siRNA-**1** is typically less potent than its phosphodiester equivalent siRNA-**2**.

4′-Thioribose modifications are well tolerated at both the 3′- and 5′-ends of the antisense strand (siRNA-**3** and siRNA-**9**, Figure 2). The 3′-end of the antisense strand has previously been modified with a variety of chemical modifiers without any observed loss in siRNA potency.[20,26,31] Since the 3′-end of the antisense strand is thought to play a minor role in siRNA recognition by the RISC complex,[7,50] it is not surprising that 4′-thioribose modifications as well as other chemical modifiers are well tolerated in this region. However, the 5′-end of the antisense strand was more sensitive to chemical modifications.[19,25−29,31] The LNA, 2′-*O*-MOE, 2′-*O*-Me, and 2′-*O*-allyl modifications when placed at the 5′-end of the antisense strand resulted in a drop in siRNA activity. However, no such negative effect on siRNA potency was observed with 4′-thioribose residues at the 5′-end of the antisense strand (siRNA-**9**, Figure 2).

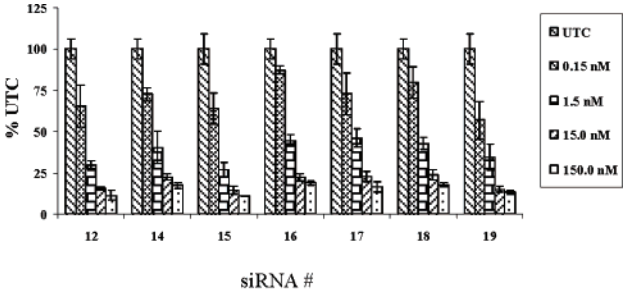
The RISC complex has been shown to recognize the ends of an siRNA duplex with a distinct 5′-antisense directionality,[50] and the drop in siRNA potency observed when 5′-termini are modified with 2′-ribose modifications has been attributed to steric interference with either duplex recognition or loading by the RISC complex.[31] Crystallography studies of model antisense strands interacting with proteins homologous to mammalian Ago2 (the putative nuclease of the RISC complex) support this model tolerating minimal modification at the 2′-position of 5′-

terminal residues of the antisense strand.[47,48] Furthermore, these structural studies indicate a substantial conformational distortion of the 5′-terminal end of the antisense strand must occur to accommodate binding to the C-terminal domain of the Ago2 protein which anchors the strand. This explains, in part, the reduced activity observed in certain circumstances, with multiple 5′-modifications such as 2′-F and LNA, which have a high degree of conformational rigidity and would not be expected to easily adopt the distorted conformation seen in the crystal structures. Since siRNAs with 4′-thioribose modifications at the 5′-end of the antisense strand show no decrease in siRNA activity, one infers that this modification is able to interact productively with the nuclease component of mammalian RISC.

When 4′-thioribose residues were placed toward the interior of the siRNA duplex, a slightly different picture emerged. siRNA-**5** (IC_{50} = 0.48 nM, Figure 2) retains siRNA activity and is 2-fold more potent than the control (siRNA-**2**, IC_{50} = 0.95 nM, Figure 2). This is in contrast to siRNA-**4**, which contains only a one-base shift of the block of three 4′-thioribose modifications and suffers a 15-fold loss in potency. siRNA-**8** (IC_{50} = 41.5 nM, Figure 2), which has residues 7−9 from the 5′-end of the antisense strand replaced with 4′-thioribonucleoside residues, leads to substantial loss (40-fold) in activity.

Elbashir et al. have shown that the primary site of RISC-mediated cleavage on the target mRNA was opposite the 10th and 11th residues on the antisense strand.[27] For the expected A-form RNA helix that is formed between the siRNA antisense strand and the target mRNA, the positions where 4′-thioribose inhibits potency are not directly across the major or minor groove from the expected cleavage site. Furthermore, they are not on the side of the duplex that makes obvious direct contacts with the Ago2 protein in a model derived from the crystal structure of the bound antisense strand.[48,62] It is therefore not readily apparent why modifying positions 7−9 or 13−15 on the antisense strand inhibits activity. It is conceivable that 4′-thioribonucleotides might have a local geometry different from that of their 4′-oxo counterparts in an siRNA duplex in a manner not evident from the structural data available to date. However, we did not observe any gross deviations from A-form helical geometry (CD spectroscopy, data not shown), so it would have to be a subtle effect. Also, Haeberli et al.[63] have recently described the crystal structure of an RNA duplex containing 4′-thiocytidine residues without noting any significant differences in helix conformation around the 4′-thioribose residues. Also, there were no significant changes in the hydration of the RNA backbone and the minor groove.

Next we placed 4′-thioribose residues at both the 3′- and 5′-ends of the antisense strand. This is referred to as the "gapmer" design represented by siRNA-**10** and siRNA-**13** (Figure 3). The gapmer design was chosen as it is used extensively in antisense therapeutics to protect the ends of the oligonucleotide from exonuclease cleavage.[64] siRNA-**10** and siRNA-**13** (IC_{50} = 5.9 and 1.2 nM, respectively, Figure 3) exhibit respectable siRNA activity. However, siRNA-**10**, which has four 4′-thioribonucleoside residues at each end of the antisense strand, is about 6-fold less potent than the parent siRNA-**2** (IC_{50} = 0.95 nM, Figure 3). siRNA-**13**, which has only two 4′-thioribonucleoside residues at both ends, is only about 3-fold less potent than the unmodified siRNA-**12** (IC_{50} = 0.4 nM), indicating a definite inverse relationship between the number of 4′-thioribose modifications in the antisense strand and siRNA activity. Taken together, these results suggest that not only the number but also the placement of 4′-thioribose modifications within the antisense strand has implications for siRNA activity. Simply increasing the number



siRNA No.		Sequence			IC_{50} (nM)
siRNA-12	5′	AAGUAAGGACCAGAGACAA	3′	s	0.4
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-14	5′	AAGUAAGGA**CC**AGAGA**C**AA	3′	s	1.1
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-15	5′	**AA**GUAAGGACCAGAGAC**AA**	3′	s	0.28
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-16	5′	AAGUAAGGA**CC**AGAGA**C**AA	3′	s	2.4
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-17	5′	**AA**GUAAGGACCAGAGAC**AA**	3′	s	1.4
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-18	5′	AAGUAAGGA**CC**AGAGA**C**AA	3′	s	1.7
	3′	**UU**CAUUCCUGGUCUCUG**UU**	5′	as	
siRNA-19	5′	**AA**GUAAGGACCAGAGAC**AA**	3′	s	0.21
	3′	**UU**CAUUCCUGGUCUCUG**UU**	5′	as	

Figure 4. Positional effects of 4′-thioribonucleoside residues in the sense and antisense strands on siRNA activity. Dose-dependent reduction of PTEN mRNA in HeLa cells by siRNA duplexes containing 4′-thioribonucleoside residues in the sense strand. Underlined sequences have a phosphorothioate backbone. The 4′-thioribonucloeside residues are indicated in red. UTC = untreated control.

of 4′-thioribose modifications can result in decreased siRNA activity as has been reported previously,[42] while optimized placement can actually enhance siRNA potency as seen for siRNA-**5**, siRNA-**7**, and siRNA-**9**.

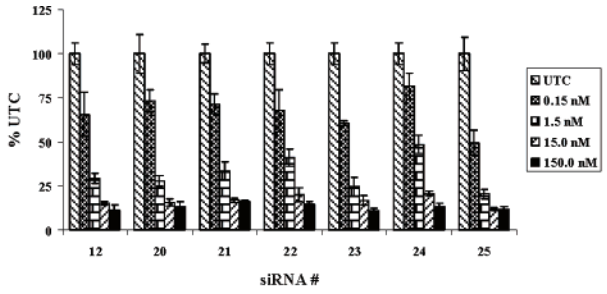
Sense Strand SAR. siRNA duplexes containing 4′-thioribonucleoside residues in the sense strand (siRNA-**14** to siRNA-**19**, Figure 4) show good activity in reducing PTEN mRNA levels in HeLa cells. Dispersing these residues in the interior of the sense strand (siRNA-**14**) has a slight negative effect on siRNA potency (IC_{50} = 1.1 nM vs IC_{50} = 0.4 nM). Pairing with a phosphorothioate antisense strand (siRNA-**16**, IC_{50} = 2.4 nM) causes a further loss in potency as observed previously (Figure 2, siRNA-**1**, IC_{50} = 1.6 nM, vs siRNA-**2**, IC_{50} = 0.95 nM). Activity is regained when the dispersed motif is replaced with the gapmer motif (siRNA-**15**, IC_{50} = 0.28 nM), once again highlighting the utility of an optimized gapmer design. This trend is confirmed when one compares siRNA-**16** (IC_{50} = 2.4 nM) with siRNA-**17** (IC_{50} = 1.4 nM). siRNA-**19**, in which both the 3′- and 5′-termini of the sense and the antisense strands are made up of 4′-thioribonucleoside residues (gapmer siRNA), is one of the most potent siRNAs in this study, having an IC_{50} of 0.21 nM. We attribute this to the stabilization of the ends of the siRNA toward exonuclease degradation.

Combined Effect of 4′-Thioribose and 2′-Ribose Modifications on siRNA Activity. During the course of designing 4′-

thioribose-modified motifs, we soon realized that we would not achieve the desired degree of serum stability by modifying only one strand at a time. We believe the construct designs reported recently[43] that have only one strand modified will face similar limitations in therapeutic application. Our preliminary experiments showed that the best motif for 4′-thioribose modification is the one that will have both strands end modified (Figure 4). As both our data and the literature report[43] indicate that there is a threshold of the number of 4′-thioribose residues in an siRNA duplex, we envisaged that combining other nuclease-resistant RNA modifications with 4′-thio-RNA should lead to superior siRNA designs. The 2′-modified RNAs are known to be resistant to nucleases and as such have been candidates for siRNA applications. We have recently reported an extensive SAR of 2′-ribose-modified siRNAs containing nuclease-resistant 2′-*O*-Me and 2′-*O*-MOE residues in the sense and/or antisense strands, where we showed that both modifications were well tolerated at the 3′- and 5′-ends of the sense strand of siRNA.[31] In the same report, we also demonstrated that both these modifications were not well tolerated at the 5′-end of the antisense strand while the 2′-*O*-Me modification was better tolerated at the 3′-end of the antisense strand. These data suggest that using bulkier 2′-substituted ribose residues in the sense strand or 3′-end of the antisense strand should not interfere with siRNA activity. This design should also lead to a favorable bias for loading the guide antisense strand into the RISC.

The placement of bulky residues in combination with the 4′-thioribose modification was optimized in the designs shown in Figure 5. As expected, siRNA-**21** through siRNA-**25** generally showed potent siRNA activity, demonstrating the compatibility of the 4′-thioribose with 2′-ribose modifications. siRNA-**21** (IC_{50} = 0.66 nM) and siRNA-**22** (IC_{50} = 0.78 nM) show slightly reduced potency relative to unmodified siRNA-**12** (IC_{50} = 0.4 nM). Next, by optimizing the placement of bulky 2′-modified residues in combination with the 4′-thioribose modification, we introduced minimal steric bulk at the 5′-end of the antisense strand while increasing it at the 3′-end (siRNA-**23** to siRNA-**25**, Figure 5). These designs showed improved potency over the parent siRNA (siRNA-**12**), exhibiting a distinct additive effect of these designs on siRNA activity. siRNA-**24** showed reduced potency (IC_{50} = 1.9 nM), which we attribute to a combination of reduced nuclease resistance resulting from the exposed unmodified ends of the sense strand. Thus, the 4′-thioribose modification is generally well tolerated in both the sense and antisense strands of siRNA duplexes, and is also compatible with other nuclease-stabilizing 2′-modifications. Importantly, by using optimized chemically modified siRNA constructs (siRNA-**23** and siRNA-**25**, Figure 5), we can perhaps achieve a degree of selectivity in the RISC strand-loading process which results in enhanced siRNA potency.

Thermal Stability of 4′-Thioribose-Modified siRNA Duplexes. Because of the known increase in the thermal stability of 4′-thio-RNA-containing duplexes, and the literature reports suggesting that decreasing the stability of the end of the duplex containing the 5′-end of the antisense strand increases its propensity to be loaded into the RISC complex,[49,50] we evaluated the thermal and plasma stability of selected siRNA duplexes (Table 1). The 4′-thioribonucleoside residues caused a slight increase in the thermal stability of siRNA duplexes (0.3 °C per residue) when present either in the sense or antisense strands. This is in agreement with the previously reported thermodynamic stability of 4′-thio-RNA duplexes.[38−42] The 2′-*O*-MOE modification is known to provide a significant enhancement in thermal stability to oligonucleotides. Indeed, siRNA-**20** with a



siRNA No.		Sequence			IC_{50} (nM)
siRNA-12	5′	AAGUAAGGACCAGAGACAA	3′	s	0.4
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-20	5′	*AAG*UAAGGACCAGAGA*CAA*	3′	s	0.58
	3′	UUCAUUCCUGGUCUCUGUU	5′	as	
siRNA-21	5′	*AAG*UAAGGACCAGAGA*CAA*	3′	s	0.66
	3′	**UU**CAUUCCUGGUCUCUG**UU**	5′	as	
siRNA-22	5′	AAGUAAGGACCAGAGACAA	3′	s	0.78
	3′	*UUC*AUUCCUGGUCUCUG**UU**	5′	as	
siRNA-23	5′	*AAG*UAAGGACCAGAGA*CAA*	3′	s	0.2
	3′	*UUC*AUUCCUGGUCUCUG**UU**	5′	as	
siRNA-24	5′	AAG**U**AAGGA**CC**AGAGA**C**AA	3′	s	1.9
	3′	*UUC*AUUCCUGGUCUCUG**UU**	5′	as	
siRNA-25	5′	**AA**GUAAGGACCAGAGAC**AA**	3′	s	0.15
	3′	*UUC*AUUCCUGGUCUCUG**UU**	5′	as	

Figure 5. Effects of 4′-thioribonucleoside residues in combination with 2′-modifications on siRNA activity. Dose-dependent reduction of PTEN mRNA in HeLa cells by siRNA duplexes containing 4′-thioribose residues in combination with 2′-*O*-Me- or 2′-*O*-MOE-modified residues. The 4′-thioribose, 2′-*O*-Me-modified, and 2′-*O*-MOE-modified nucleoside residues are indicated in red, blue (italics), and orange (italics), respectively. UTC = untreated control.

2′-*O*-MOE-modified sense strand at the 3′- and 5′-termini exhibited improved thermal stability (T_m = 74.3 °C, Table 1) relative to the unmodified siRNA-**12** (T_m = 72.8 °C, Table 1). Interesting enough, the thermal stability was further enhanced when 4′-thioribonucleoside substitutions were combined with 2′-substitutions, as in siRNA-**21** and siRNA-**25** (T_m = 75.5 and 75.4 °C, Table 1). Thus, the thermal stabilizing effect of the 4′-thioribonucleoside residues works additively with 2′-modifications. It is especially interesting in light of the literature, which reports increased activity of duplexes having a destabilized 5′-antisense strand end, to note that our duplexes having increased overall T_m do not show decreased potency. We made no attempt to intentionally destabilize one end of the duplex, as we felt that added thermal stability may also be an important factor in the stability of the duplex to degradation by nucleases, as the duplex appears to aid in protection from degradation by single-stranded nucleases. It is reasonable to predict that increased rates of "breathing" by the ends of the duplex would increase degradation as the single-strand region of RNA is exposed.

Plasma Stability of 4′-Thioribose-Modified siRNAs. As expected, we found that chemical optimization led to significant improvement of the plasma stability profiles for this series (Table 1) of siRNAs. On the basis of their respective half-lives

Table 1. Thermal and Plasma Stability and siRNA Activity of Selected siRNA Duplexes Containing 4′-Thioribose (Red) Residues in Combination with 2′-*O*-Me-Modified (Blue Italics) or 2′-*O*-MOE-Modified (Orange Italics) Residues[a]

siRNA No.	Sequence*			*T*m °C	$t_{1/2}$[a] h	IC_{50} (nM)
siRNA-12	5′	AAGUAAGGACCAGAGACAA	3′	72.8	~1	0.4
	3′	UUCAUUCCUGGUCUCUGUU	5′			
siRNA-18	5′	AAGUAAGGACCAGAGACAA	3′	75.8	2.5	1.7
	3′	UUCAUUCCUGGUCUCUGUU	5′			
siRNA-19	5′	AAGUAAGGACCAGAGACAA	3′	76.8	14	0.21
	3′	UUCAUUCCUGGUCUCUGUU	5′			
siRNA-20	5′	*AAG*UAAGGACCAGAGA*CAA*	3′	74.3	3	0.58
	3′	UUCAUUCCUGGUCUCUGUU	5′			
siRNA-21	5′	*AAG*UAAGGACCAGAGA*CAA*	3′	75.5	13	0.66
	3′	UUCAUUCCUGGUCUCUGUU	5′			
siRNA-25	5′	AAGUAAGGACCAGAGACAA	3′	75.4	18	0.15
	3′	*UUC*AUUCCUGGUCUCUGUU	5′			

[a] Notes: *, siRNA duplexes, sense strand 5′ to 3′, antisense strand 3′ to 5′; a, $t_{1/2}$ = plasma half-life; thermal melting and plasma (25% heparinized mouse plasma) stability studies were carried out as described in the Experimental Section.

in 25% heparinized mouse plasma, siRNA-**18**, siRNA-**19**, siRNA-**21**, and siRNA-**25** were approximately 2.5-, 14-, 13-, and 18-fold more resistant to degradation than unmodified siRNA-**12**. This observation is in agreement with the known nuclease stability of 4′-thioribonucleotides.[38−42] The need to protect the ends of individual strands is obvious when one compares siRNA-**18** and siRNA-**19**. siRNA-**18**, in which the unmodified 3′- and 5′-ends of the sense strand are exposed to serum nucleases, has a half-life of 2.5 h. When these exposed ends are blocked by incorporation of 4′-thioribose residues (siRNA-**19**), the plasma half-life increases to 14 h. It should however be noted that siRNA-**18** is still 2.5 times more stable in mouse plasma than unmodified siRNA-**12** probably due to stabilization of the duplex toward endonucleolytic cleavage. These differences in plasma half-life between siRNA-**19** and siRNA-**18** indicate that exonucleases and not endonucleases are primarily responsible for siRNA degradation in mouse plasma. In addition to the benefit provided by 4′-thioribose "gapmers" in each strand, the strategic placement of 4′-thioribose and 2′-*O*-Me and 2′-*O*-MOE modifications also greatly enhanced the plasma stability of siRNA. These modifications, when used in combination at the ends of the duplex (siRNA-**21** and siRNA-**25**), show significant gains in plasma stability over unmodified control siRNA-**12**. These data support the hypothesis that degradation in plasma is occurring by nucleases which attack the ends of the construct most likely when they are exposed as single strands due to breathing of the duplex. It is remarkable that as few as two 4′-thioribonucleoside residues at each end of both strands provide greatly enhanced protection from nucleolytic degradation without compromising the intrinsic potency of the siRNA mechanism.

Conclusion

We have evaluated the effect of the 4′-thioribonucleoside modification on the RNAi activity of siRNAs, individually and in combination with 2′-*O*-Me and 2′-*O*-MOE modifications. The siRNAs containing 4′-thioribonucleoside substitutions were highly effective in reducing target PTEN mRNA in HeLa cells. Moreover, we showed that, by carefully positioning and/or combining 4′-thioribonucleosides with 2′-ribose modifications, siRNA potency could be increased beyond that of unmodified siRNAs (siRNA-**7**, siRNA-**9**, siRNA-**19**, siRNA-**23**, and siRNA-**25** are 2−4-fold more potent than the corresponding unmodified siRNAs). Importantly, the improved potency of siRNA-**23** and siRNA-**25** demonstrates that modified chemistries with differential tolerances for substitutions at key positions in the duplex such as the 5′-end of the antisense strand can be employed in consideration of the biased strand-loading hypothesis reported for the RISC complex[49,50] to rationally design potent siRNAs.

Our results also demonstrate the success of our positional SAR strategy in identifying plasma-stable siRNAs. We have confirmed that 4′-thioribose modifications when not optimally placed lead to a moderate increase in plasma stability (siRNA-**18**). Using a rational design strategy (gapmer design, siRNA-**19**) and a combination of modified chemistries (siRNA-**21** and siRNA-**25**), we have identified constructs that are simultaneously 2−4-fold more potent and 13−18-fold more stable than unmodified siRNAs. This observed plasma stability and potent siRNA activity of our modified siRNA constructs will be valuable when elucidating an SAR for systemically administered siRNAs in vivo, where unmodified siRNAs are likely to be too unstable to be therapeutically viable. The potency, plasma stability, and compatibility of the 4′-thioribose modification with other chemical modifications demonstrated herein provide opportunities to address these questions in animal studies to further optimize siRNAs for therapeutic applications.

Importantly, the 4′-thioribose modification adequately compensates for the inability to utilize highly stabilizing 2′-ribose modifications at the 5′-end of the antisense strand. Furthermore, the incorporation of sulfur into the siRNA duplex without incurring the loss of potency observed with phosphorothioate siRNAs in a non-phosphorothioate context raises the possibility of influencing tissue distribution and in vivo potency in much the same way as sulfur-containing antisense oligonucleotides. Further studies to fully exploit these potential benefits of 4′-thio-siRNAs are currently under way in our laboratory, and the results will be published in due time.

Experimental Section

General Procedures. Solvents used were of anhydrous grade and were stored under nitrogen at all times. The RNA phosphoramidites with a 2′-*O*-(*tert*-butyldimethylsilyl) protecting group and reagents were procured form Glen Research Inc., Virginia. All other starting materials and reagents were purchased from Aldrich Chemical Co. and were used without further purification. Thin-layer chromatography was performed on precoated plates (silica gel 60 F254, EM Science, New Jersey) and visualized with UV light and spraying with a solution of *p*-anisaldehyde (6 mL), H_2SO_4 (8.3 mL), and CH_3COOH (2.5 mL) in C_2H_5OH (227 mL) followed by charring. ^1H NMR spectra were referenced using internal standard $(CH_3)_4Si$ and ^{31}P NMR spectra using external standard 85% H_3PO_4. Mass spectra were recorded by Mass Consortium, San Diego, CA, and the College of Chemistry, University of California, Berkeley, CA.

(*R*)-1,4-Anhydro-2-*O*-(2,4-dimethoxybenzoyl)-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-4-sulfinyl-D-ribitol (2). Diethyl L-tartrate (60 mL, 200.16 mmol) was added to a vigorously stirring solution of titanium(IV) isopropoxide (30 mL, 50.2 mmol) in dry dichloromethane (200 mL), resulting in a straw-yellow-colored solution. After being stirred at room temperature, the solution was cooled to −20 °C, and *tert*-butyl hydroperoxide (40 mL of a 6.0

M solution in decane) was added. After a few minutes a solution of **1** (70 g, 126 mmol) in dry dichloromethane (200 mL) was added. Stirring was continued at the same temperature for 18−24 h till TLC showed no presence of **1**. The reaction was quenched by the addition of water and allowed to come to room temperature. The resulting white precipitate was filtered through a Celite plug, and the cake was washed exhaustively with pentanes. The filtrate was transferred to a separating funnel, washed three times with water and once with brine, and dried over anhydrous sodium sulfate. Solvents were removed under reduced pressure, and the residue was purified by flash chromatography (1:1 ethyl acetate/hexanes) to give **2** (66.4 g, 116 mmol) in 92% isolated yield as a single isomer: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.92 (d, 1 H, $J = 8.3$ Hz), 6.49 (m, 2 H), 5.79 (dd, 1 H, $J_1 = 5.4$ Hz, $J_2 = 3.6$ Hz), 4.59 (d, 1 H, $J = 12.8$ Hz), 4.22 (dd, 1 H, $J_1 = 2.8$ Hz, $J_2 = 12.8$ Hz) 4.12 (dd, 1 H, $J_1 = 3.6$ Hz, $J_2 = 12.0$ Hz), 3.88 (s, 3 H), 3.86 (s, 3 H), 3.57 (dd, 1 H, $J_1 = 5.4$ Hz, $J_2 = 15.5$ Hz), 3.49 (dd, 1 H, $J_1 = 12.0$ Hz, $J_2 = 2.8$ Hz), 2.89 (d, 1 H, $J = 15.5$ Hz), 1.11−0.94 (m, 28 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 164.5, 164.1, 161.6, 134.0, 111.7, 104.5, 98.8, 77.2, 72.5, 72.4, 67.9, 55.8, 55.4, 54.3, 17.2, 17.1, 17.1,17.0, 17.0, 16.8, 16.8, 13.28, 13.0, 12.6, 12.5; HRMS (FAB) *m*/*z* calcd for $C_{26}H_{45}O_8SSi_2^+$ 572.2295, found 573. 2296.

1-[2,3-*O*-Bis(*tert*-butyldimethylsilyl)-5-*O*-(4,4′-dimethoxytrityl)-4-thio-β-D-ribofuranosyl)]uracil (10). 9[41] (9.2 g, 13.6 mmol) and imidazole (6 g, 81.6 mmol) were dissolved in anhydrous pyridine (30 mL) and the resulting solution cooled in an ice bath. (TBS)Cl (4.1 g, 27.2 mmol) was added and the reaction mixture stirred at room temperature for 24 h, at which point no more starting material was detected by TLC (SiO_2, 25% ethyl acetate in dichloromethane). The reaction was quenched by addition of ice and partitioned between ethyl acetate (200 mL) and water (200 mL). The ethyl acetate layer was washed with saturated sodium bicarbonate solution (3 × 100 mL), ice-cold 10% citric acid solution (100 mL), and brine (20 mL). After the layer was dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure and the residue was dried to a white foam in vacuo over phosphorus pentoxide. This material was used as such in the next step without further purification.

***N*^{4}-Benzoyl-1-[-5-*O*-(4,4′-dimethoxytrityl)-4-thio-β-D-ribofuranosyl)]cytosine (11).** In an oven-dried reaction vessel, 1,2,4-triazole (19.6 g, 283.36 mmol) was suspended in anhydrous acetonitrile (200 mL) and the resulting suspension cooled to 0 °C in an ice bath. Phosphorus oxychloride (7.55 mL, 80.96 mmol) was added dropwise to the suspension followed by triethylamine (56.5 mL, 404.8 mmol) as a solution in dry acetonitrile (100 mL). The resulting suspension was stirred at 0 °C for 30 min. Compound **10** (16 g, 20.24 mmol) was dissolved in anhydrous acetonitrile (100 mL) and the resulting solution added dropwise to the reaction mixture. The resulting pale yellow suspension was stirred overnight at room temperature under an inert atmosphere. The reaction mixture was then partitioned between ethyl acetate (500 mL) and saturated sodium bicarbonate solution (500 mL). The ethyl acetate layer was washed with brine (20 mL) and dried over anhydrous sodium sulfate. The solvents were removed under reduced pressure, and the residue was dissolved in 1,4-dioxane (100 mL). Aqueous ammonia (28−30 wt % solution, 50 mL) was added, and the mixture was stirred in a sealed flask overnight. The reaction mixture was partitioned between ethyl acetate (300 mL) and water (500 mL). The ethyl acetate layer was washed with brine (20 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residue was dried overnight in vacuo over phosphorus pentoxide. To this was added anhydrous pyridine (50 mL), followed by benzoic anhydride (9.15 g, 40.48 mmol), and the mixture was stirred under an inert atmosphere overnight. The reaction mixture was partitioned between ethyl acetate (300 mL) and saturated sodium bicarbonate solution (200 mL). The ethyl acetate layer was washed twice with saturated sodium bicarbonate solution (2 × 200 mL), once with ice-cold 10% citric acid solution (100 mL), and with brine (20 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residue was dried in vacuo over phosphorus pentoxide. The residue was then dissolved in anhydrous tetrahydrofuran (100 mL). Triethylamine (10 mL) and triethylamine trihydrofluoride (20 mL) were added, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate (300 mL) and transferred to a separating funnel. The ethyl acetate layer was washed with saturated sodium bicarbonate solution (3 × 100 mL) and brine (10 mL) and dried over anhydrous sodium sulfate. The residue obtained was purified by flash silica gel column chromatography (5% methanol, 0.1% triethylamine in dichloromethane) to yield **11** (13 g, 96% yield): ^{1}H NMR spectra in agreement with published literature values;[40] HRMS (FAB) *m*/*z* calcd for $C_{37}H_{36}N_3O_7S^+$ 666.2196, found 666.2204.

***N*^{4}-Benzyol-1-[2-*O*-(*tert*-butyldimethylsilyl)-5-*O*-(4,4′-dimethoxytrityl)-4-thio-β-D-ribofuranosyl)]cytosine (13).** Compound **11** (10 g, 15 mmol) was dissolved in anhydrous tetrahydrofuran (150 mL) containing pyridine (3 mL). Silver nitrate (3.2 g, 18.75 mmol) was added and the reaction mixture stirred for 30 min at room temperature under an inert atmosphere. *tert*-Butyldimethylsilyl chloride (2.83 g, 18.75 mmol) was added, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was filtered through a Celite plug and partitioned between ethyl acetate (300 mL) and saturated sodium bicarbonate solution (100 mL). The ethyl acetate layer was washed with brine (10 mL) and dried over anhydrous sodium sulfate. Solvent was removed under reduced pressure, and the residue was purified by flash silica gel column chromatography (25% ethyl acetate, 0.1% triethylamine in dichloromethane) to yield **12** (5.5 g, 47%) and **13** (6.0 g, 51.3%). The isolated **12** was converted to **13** by being stirred in dry ethanol (50 mL) containing 1% triethylamine and 0.1% imidazole to give a 1:1 mixture of **12** and **13**. The desired isomer **13** was separated by flash silica gel column chromatography as before, and the procedure was repeated till almost all of **12** was converted to **13** (9.95 g) with a final isolated yield of 85%: ^{1}H NMR spectra in agreement with published literature values;[41,44] HRMS (FAB) *m*/*z* calcd for $C_{43}H_{50}N_3O_7SSi^+$ 780.3060, found 780.3143 $[M + H]^+$.

***N*^{4}-Benzyol-1-[2-*O*-(*tert*-butyldimethylsilyl)-3-*O*-[(2-cyanoethyl)(*N*,*N*-diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-4-thio-β-D-ribofuranosyl)]cytosine (14).** Compound **14** was synthesized using a reported procedure.[59] Compound **13** (5.4 g, 6.93 mmol) and tetrazole (294 mg, 1.35 mmol) were dissolved in anhydrous DMF (40 mL) under an inert atmosphere. *N*-Methylimidazole (107 μL, 1.35 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (3.3 mL, 10.4 mmol) were added. The reaction mixture was stirred overnight at room temperature and partitioned between ethyl acetate (200 mL) and saturated sodium bicarbonate solution (50 mL). The ethyl acetate layer was washed with water (2 × 300 mL) and brine (10 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue purified by flash silica gel column chromatography (25% ethyl acetate, 0.1% triethylamine in dichloromethane) to yield **14** (6.4 g, 94%): ^{1}H and ^{31}P NMR spectra were agreement with published literature values;[40,43] HRMS (FAB) *m*/*z* calcd for $C_{52}H_{67}N_5O_8PSSi^+$ 980.4139, found 980.4199.

RNA Synthesis. RNA oligonucleotides were synthesized on a solid-phase DNA/RNA synthesizer using the 2′-*O*-TBS-RNA phosphoramidites (TBS = *tert*-butyldimethylsilyl) according to the reported protocols.[38] The 4′-thio, 2′-*O*-Me, and 2′-*O*-MOE phosphoramidites with exocyclic amino groups protected with benzoyl (Bz for A and C) or isobutyryl (ibu for G) were used for the synthesis of the RNA chimera. A 0.12 M solution of the phosphoramidites in anhydrous acetonitrile was used for the synthesis. Oxidation of the internucleosidic phosphite to the phosphate was carried out using *tert*-butyl hydroperoxide/acetonitrile/water (10:87:3) with a 10 min oxidation time. 3*H*-1,2-Benzodithiol-3-one 1,1-dioxide[57] (Beaucage reagent, 0.15 M solution in anhydrous acetonitrile) was used as the sulfur-transfer agent for the synthesis of oligoribonucleotide phosphorothioates. Chemical phosphorylation reagent procured from Glen Research Inc. was used to phosphorylate the 5′-terminus of modified oligonucleotides. Samples of 12 equiv of the phosphoramidite solutions were delivered in two

portions, each followed by a 6 min coupling wait time. All other steps in the protocol supplied by the manufacturer were used without modification. The stepwise coupling efficiencies were more than 97%. After completion of the synthesis, the solid support was treated with triethylamine/acetonitrile (1:1) for 30 min. Then it was suspended in a mixture of aqueous ammonium hydroxide (28−30 wt %)/ethanol (3:1) and the suspension heated at 55 °C for 6 h to complete the removal of all protecting groups except the TBS group at the 2′-position. The solid support was filtered, and the filtrate was concentrated to dryness. The residue obtained was resuspended in anhydrous triethylamine trihydrofluoride/triethylamine/1-methyl-2-pyrrolidinone solution (0.75 mL of a solution of 1 mL of triethylamine trihydofluoride, 750 μL of triethylamine, and 1.5 mL of 1-methyl-2-pyrrolidine, to provide a 1.4 M HF concentration) and the suspension heated at 65 °C for 1.5 h to remove the TBS groups at the 2′-position.[36] The reaction was quenched with 1.5 M ammonium bicarbonate (0.75 mL), and the mixture was loaded onto a Sephadex G-25 column (NAP Columns, Amersham Biosciences Inc.). The oligonucleotides were eluted with water, and the fractions containing the oligonucleotides were pooled together and purified by high-performance liquid chromatography (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}, solvent A = 100 mM ammonium acetate, 30% aqueous acetonitrile, solvent B = 1.5 M NaBr in A, 0−40% B in 60 min, flow rate 1.5 mL min^{-1}, λ = 260 nm). Fractions containing full-length oligonucleotides were pooled together (assessed by capillary gel electrophoresis (CGE) analysis, >90%) and evaporated. The residue was dissolved in sterile water (0.3 mL), absolute ethanol (1 mL) was added, the resulting mixture was cooled to −20 °C for 1 h, and the precipitate formed was pelleted out by centrifugation (NYCentrifuge 5415C, Eppendorf, Westbury, NY) at 3000 rpm. The supernatant was decanted, and the pellet was redissolved in 5 M ammonium acetate (0.3 mL) solution. Ethanol (1 mL) was added, the resulting mixture cooled to −20 °C for 1 h to get a precipitate, and the precipitate pelleted out in a centrifuge (NYCentrifuge 5415C, Eppendorf) at 3000 rpm for 15 min. The pellet was collected by decanting the supernatant. The pelleted oligonucleotides were redissolved in sterile water (0.3 mL) and precipitated by adding ethanol (1 mL) and cooling the mixture at −20 °C for 1 h. The precipitate formed was pelleted out and collected as described above. The oligonucleotides were characterized by ES MS, and the purity was assessed by capillary gel electrophoresis and HPLC (Waters, C-18, 3.9 × 300 mm, solvent A = 100 mM triethylammonium acetate, pH 7, solvent B = acetonitrile, 5−60% B in 40 min, flow rate 1.5 mL min^{-1}, λ = 260 nm).

Assembly of Modified siRNA Duplexes. Equimolar amounts of sense and antisense strands were mixed together and annealed by heating the mixture at 90 °C for 1 min and subsequently incubating it at 37 °C for 1 h. Successful duplex formation was confirmed by CGE (Beckman, MDQ CE).

Cell Culture and Transfection of Cells. HeLa cells (American Type Tissue Culture Collection, Manassas, VA) were cultured in a culture flask in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, California), 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 5000 cells/well. The cells were transfected with siRNAs complexed with 6 mg mL^{-1} Lipofectin (Invitrogen) in serum-free Opti-MEM I reduced-serum medium. The cells were incubated in the transfection medium for 4 h, the transfection medium was removed from the cells and replaced with fresh DMEM, 10% fetal calf serum, and the cells were incubated at 37 °C and 5% CO_2 for 16 h.

RNA Expression Analysis. Total RNA was harvested after 16 h using the RNeasy process from Qiagen (Valencia, California) 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). The following primer probe set (Qiagen) was used: hu PTEN (accession no. U92436.1), forward primer 5′-AATGGCTAAGTGAAGATGAC AATCAT, reverse primer 5′-TGCACATATCAT TACACCAGT-TCGT, and FAM/TAMRA probe 5′-TTGCAGCAATTCACTG-TAAAG CTGGAAAGG. Total RNA for each well was measured using RiboGreen (Molecular Probes, Eugene, OR), and these values were used for sample to sample normalization. IC_{50} values were calculated from the linear regression analysis from the plot of the logarithmic value of the siRNA concentration versus the percentage of the untreated control.

Thermal Stability Determination. The thermal stability of the modified siRNA duplexes was studied by measuring the UV absorbance versus temperature curves as described previously.[65,66] Each sample contained 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, and 4 μM sense and 4 μM antisense strands. Each T_m reported was an average of values from two experiments.

Plasma Stability Determination. Investigated siRNA constructs have been incubated at a concentration of 10 μM in 25% heparinized mouse plasma at 37 °C (Charles River Lab, Wilmingten, MA). Orotic acid was added as an internal standard (200 μM, Sigma, St. Louis, MO). At the indicated time points 10 μL aliquots were removed and quenched by treatment with proteinase K (Sigma) at 45 °C for 60 min. Samples were stored on ice until analysis by CGE utilizing a P/ACE MDQ instrument with UV detection (Beckman, Fullerton, CA). For CGE analysis the ssDNA 100-R Kit (Beckman) was used with the exception that no urea was added to the buffer. To increase sample loading, a sample-stacking procedure was performed by injecting water for 10 s at 10 psi prior to sample injection. Peak areas of the siRNA construct and orotic acid have been determined for the different time points and normalized to the peak area of orotic acid to the zero time point.

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Supporting Information Available: ^{31}P NMR and HRMS (FAB) mass spectra of compounds **7**, **8**, and **14** and ES MS and CGE profiles of selected modified oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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