Fully 2′-Modified Oligonucleotide Duplexes with Improved in Vitro Potency and Stability Compared to Unmodified Small Interfering RNA

Charles R. Allerson,*,† Namir Sioufi,‡ Russell Jarres,‡ Thazha P. Prakash,† Nishant Naik,† Andres Berdeja,§ Lisa Wanders,§ Richard H. Griffey,† Eric E. Swayze,† and Balkrishen Bhat†

Departments of Medicinal Chemistry, Antisense Lead Identification, and Research Chemistry, Isis Pharmaceuticals, 2292 Faraday Avenue, Carlsbad, California 92008

Received October 18, 2004

Abstract: We have identified a small interfering RNA (siRNA) motif, consisting entirely of 2′-*O*-methyl and 2′-fluoro nucleotides, that displays enhanced plasma stability and increased in vitro potency. At one site, this motif showed remarkable >500-fold improvement in potency over the unmodified siRNA. This marks the first report of such a potent fully modified motif, which may represent a useful design for therapeutic oligonucleotides.

The specific and reversible modulation of gene expression through the use of short synthetic oligonucleotides has proven useful in the study of gene function and as a therapeutic mechanism in man.[1] Recently, RNA interference (RNAi) has emerged as a novel mechanism that is activated in mammalian cells by small interfering RNAs (siRNAs), short RNA duplexes with strands of 21−23 nucleotides.[2,3] Once inside the cell, siRNAs associate with proteins to form an RNA-induced silencing complex (RISC).[4] A helicase activity associated with RISC separates the two strands of the duplex,[5] releasing the sense strand and permitting the binding of the antisense strand to its messenger RNA (mRNA) target. The resulting duplex is a substrate for a RISC-associated nuclease, recently identified as Ago2 (also known as eIF2C2 in man),[6] which cleaves the target transcript at a single site.[5,7]

Despite attempts to use siRNA in vivo,[8−14] the reduction of endogenous target mRNAs through the systemic delivery of siRNA has proven difficult. Although there have been conflicting reports on the nuclease stability of unmodified siRNA duplexes, there is evidence that many are degraded within minutes in mammalian serum.[9,15,16] It seems likely that siRNAs with increased nuclease stability will have a better chance at eliciting an in vivo response. Efforts to determine the optimal use of stabilizing chemistries have been the focus of several recent reports.[15,17−21] From these studies, the 2′-*O*-methyl (2′-OMe) and 2′-deoxy-2′-fluoro (2′-F) modifications have shown promise in stabilizing siRNA without disrupting the efficiency of mRNA target reduction. To further optimize the use of these and other chemistries in siRNA, we are conducting an extensive SAR analysis of chemically modified siRNA. One of the most promising designs from these studies is a fully modified duplex that consists of alternating 2′-OMe and 2′-F nucleotides. Surprisingly, duplexes with this substitution display increased in vitro potency and increased stability. Here, we report on this remarkably active motif and present our preliminary findings.

The duplexes used in our SAR study were designed to target one of two sites within the coding region of the human PTEN mRNA, both previously reported as valid target sites for siRNA.[22] Modified and unmodified duplexes were introduced into HeLa cells using a cationic lipid transfection reagent (lipofectin). In vitro activity was measured by performing RT-PCR on the PTEN mRNA and comparing to mRNA levels of untreated cells. All PTEN signals were normalized to total RNA, as measured with RiboGreen.[23]

Natural Dicer-generated siRNAs have 2-nucleotide 3′ overhangs on both ends of the short duplex.[2] In an effort to mimick this design, most synthetic siRNAs are designed with 3′ overhangs, typically with the sequence dTdT. Several recent reports have provided evidence that these overhangs bind to the PAZ domains found in numerous proteins, including the Ago2 component of RISC, perhaps functioning as a specificity determinant.[24−28] However, there have been other reports that blunt-ended RNA duplexes can function equally well and may be more stable to exonucleolytic degradation.[21] Given our goal of developing duplexes with optimal stability and activity, we explored the use of blunt-ended duplexes at both PTEN target sites (sites A and B). The activities of 19-base-pair duplexes having 3′-dTdT overhangs (**1** and **4**) were compared to those of otherwise identical blunt-ended duplexes (**2** and **5**) in a 10-point dose-response experiment (Figure 1). We found only a minimal impact on activity with the removal of the overhangs. To confirm the specificity of PTEN mRNA reduction, duplexes containing six mismatches (**3** and **6**) to the target site were included as negative controls in each experiment. Neither control affected PTEN mRNA or total RNA levels.

After validation of the use of blunt-ended constructs, the remaining SAR was performed without the use of 3′ overhangs. Among the duplex designs examined were several that belonged to a class of "alternating" motifs in which one type of modified nucleotide was placed in alternating positions with a ribonucleotide or a different modified nucleotide. In a recent publication, motifs of this type, using 2′-OMe and unmodified (2′-OH) nucleotides, were shown to have enhanced serum stability and single dose in vitro activities similar to those of the corresponding unmodified blunt-ended duplexes.[21] During the course of our studies, we examined siRNAs with the same 2′-OMe/2′-OH substitution. We found that duplexes with this motif had in vitro potencies similar to those of unmodified siRNAs (see Supporting Information). As part of our broader SAR studies, however, we also examined the effect of combining the 2′-OMe substitution with other chemistries, such as 2′-F, which has already been shown to be well-tolerated in siRNA.[15] This led to the identification of our most potent con-

* To whom correspondence should be addressed. Phone: 760-603-4697. Fax: 760-603-4654. E-mail: callerson@isisph.com.
† Department of Medicinal Chemistry.
‡ Department of Antisense Lead Identification.
§ Department of Research Chemistry.

10.1021/jm049167j CCC: $30.25
Published on Web 01/20/2005

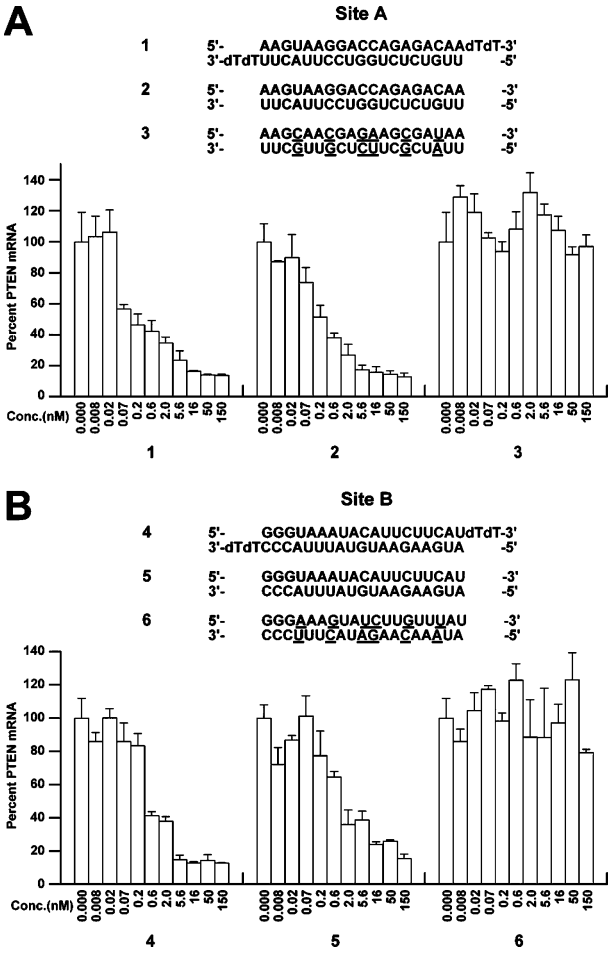

Figure 1. Reduction of endogenous PTEN mRNA in HeLa cells by siRNAs having 3′-dTdT overhangs or blunt ends. HeLa cells were transfected with siRNAs at the indicated concentrations in the presence of lipofectin and treated for 20 h followed by lysis and RT-PCR. Message levels are reported as percent of PTEN mRNA from untreated cells. The bottom strand of each duplex is complementary to the target mRNA (mismatches to the target site are indicated with an underline). (A) PTEN mRNA reduction by siRNAs targeted to site A. Cells were also treated with a duplex containing six mismatches to the target site as a negative control (**3**). (B) PTEN mRNA reduction by siRNAs targeted to site B. Cells were also treated with a duplex containing six mismatches to the target site as a negative control (**6**).

struct design, in which both strands were substituted with alternating 2′-OMe and 2′-F nucleotides (Figure 2). From an eight-point dose-response analysis in HeLa cells, we were able to estimate IC_{50} values of each duplex (Table 1; see also Supporting Information).

At site A, the duplex with alternating 2′-F/2′-OMe chemistry (**7**, Figure 2) had biological activity that was roughly equivalent to that of the parent siRNA (**2**). At site B, however, the 2′-F/2′-OMe duplex (**10**) displayed dramatically improved potency. Even at the lowest concentration (2 pM), target reduction was greater than 75% relative to untreated control, minimally reflecting 500-fold improvement in potency over the unmodified siRNA (**5**). Throughout these studies, we were cognizant of the importance of the 5′-phosphates normally present on Dicer-generated siRNA duplexes. The 5′-phosphate on the antisense strand has been shown to be critical for efficient assembly and activation of RISC in mammalian systems.[29] However, it has been shown that

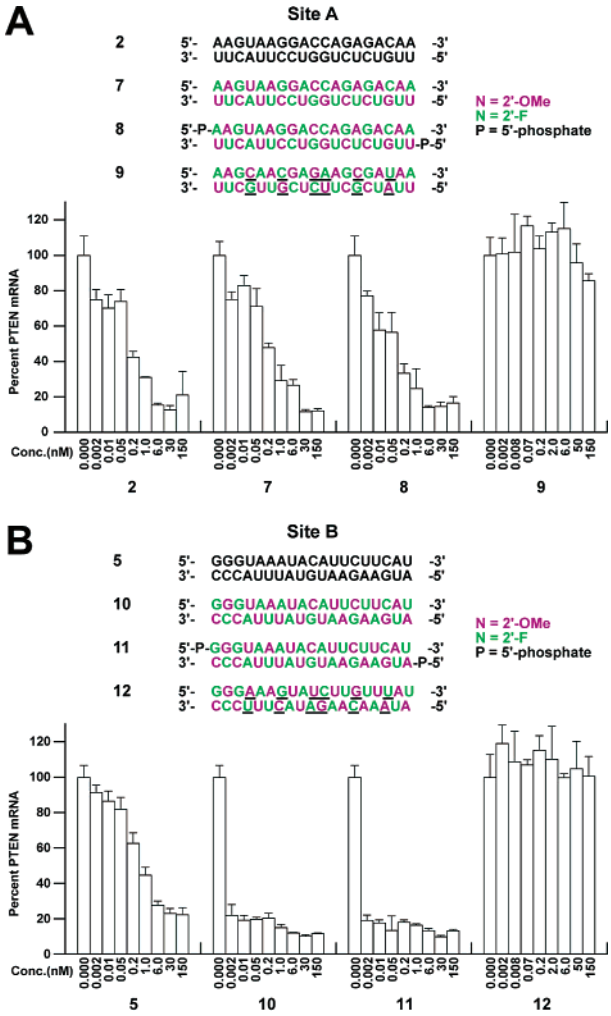

Figure 2. Reduction of endogenous PTEN mRNA in HeLa cells by unmodified or fully 2′-F/2′-OMe modified 19-base-pair oligonucleotide duplexes. HeLa cells were transfected with siRNAs at the indicated concentrations in the presence of lipofectin and treated for 20 h followed by lysis and RT-PCR. Message levels are reported as percent of PTEN mRNA from untreated cells. The bottom strand of each duplex is complementary to the target mRNA (mismatches to the target site are indicated with an underline). The 2′-F modification is indicated in green, the 2′-OMe modification is indicated in purple, while the presence of a 5′-phosphate is indicated by the letter "P". (A) PTEN mRNA reduction by duplexes targeted to site A. (B) PTEN mRNA reduction by duplexes targeted to site B.

Table 1. Summary of in Vitro Activity, Plasma Stability, and Duplex Thermal Stability[a]

duplex	site	IC_{50} (nM)	plasma half-life (min)	T_m (°C)
2	A	0.26	~30	72.8
7	A	0.31	nd	93.9
8	A	0.068	>420	nd
5	B	0.81	nd	62.0
10	B	<0.002	nd	nd
11	B	<0.002	>420	82.0

[a] nd = not determined.

prephosphorylation is generally unnecessary because the siRNAs are phosphorylated by endogenous kinases. Anticipating that chemically modified duplexes might be less efficiently processed by these kinases,[30] we compared the activities of 2′-F/2′-OMe duplexes with preestablished 5′-phosphates (Figure 2, **8** and **11**). At site A, addition of a synthetic 5′-phosphate produced

modest improvement (5-fold) in potency (Table 1). Given the already potent activity of **10**, we were unable to resolve any beneficial effect of 5′-phosphorylation at site B. Similar effects from phosphorylation have been observed with this motif on other targets, where modestly active duplexes show improvement of in vitro potency upon addition of the 5′-phosphate, while the effect on extremely potent duplexes is difficult to resolve (data not shown). Furthermore, the 5′-phosphate on the antisense strand appears to be largely responsible for this improvement in potency, although the magnitude of the effect may depend on cell type (data not shown). We also prepared and tested modified duplexes that contained six mismatches to either of the target sites (**9** and **12**). Neither mismatch-containing duplex produced a significant reduction in PTEN mRNA or total RNA.

To examine the relative serum stabilities of these duplexes, we tested unmodified siRNA **2** and 2′-F/2′-OMe modified duplexes **8** and **11** for their ability to resist degradation in mouse plasma. Each of the duplexes was treated with 25% mouse plasma at 37 °C for up to 7 h. At various time points, aliquots were removed and examined for intact duplex by capillary gel electrophoresis.[31,32] From these measurements, we plotted percent intact duplex against time and assessed the relative stabilities of the three duplexes (Figure 3A, Table 1). From this straightforward analysis, the stability of the 2′-F/2′-OMe duplexes was striking. Even after 7 h, the duplexes were greater than 60% (**8**) or 70% (**11**) intact. While there was an initial loss of duplex in each case, this may correspond to rapid loss of imperfectly annealed strands. Regardless, the remaining duplex degrades at a rate that suggests a half-life of much greater than 7 h.

It has previously been shown that the use of 2′-F modifications can increase the thermal stability of oligonucleotide duplexes.[33] Because duplexes **7**, **8**, **10**, and **11** each contain a total of 19 2′-F nucleotides, we anticipated that these duplexes might have much greater thermal stabilities than their unmodified counterparts. To examine this possibility, we measured the thermal stabilities of two control duplexes (**2** and **5**) and two modified duplexes (**7** and **11**) (Figure 3B). In each case we see a roughly 20 °C increase in T_m with the fully modified duplexes. This corresponds to slightly more than 1 °C increase in T_m per 2′-F substitution. This increase in thermal stability is likely to explain in part the enhanced plasma stability of the modified duplexes and may improve the interaction between the antisense strand and the target mRNA.

It is surprising that duplexes with such high thermal stabilities can function so efficiently and potently relative to the unmodified siRNAs. Recent studies have suggested that RISC chooses which strand to retain on the basis of differences in thermodynamic stability at the 5′ ends of the strands, with the strand having the 5′ end of lower stability being more likely to load into RISC.[34,35] It is worth noting that one end of the site B duplex contains three consecutive G−C base pairs. Although the introduction of 2′-F nucleotides should raise the thermodynamic stability of both ends of the duplex, it may increase the already more-stable end above a critical threshold that the RISC-associated

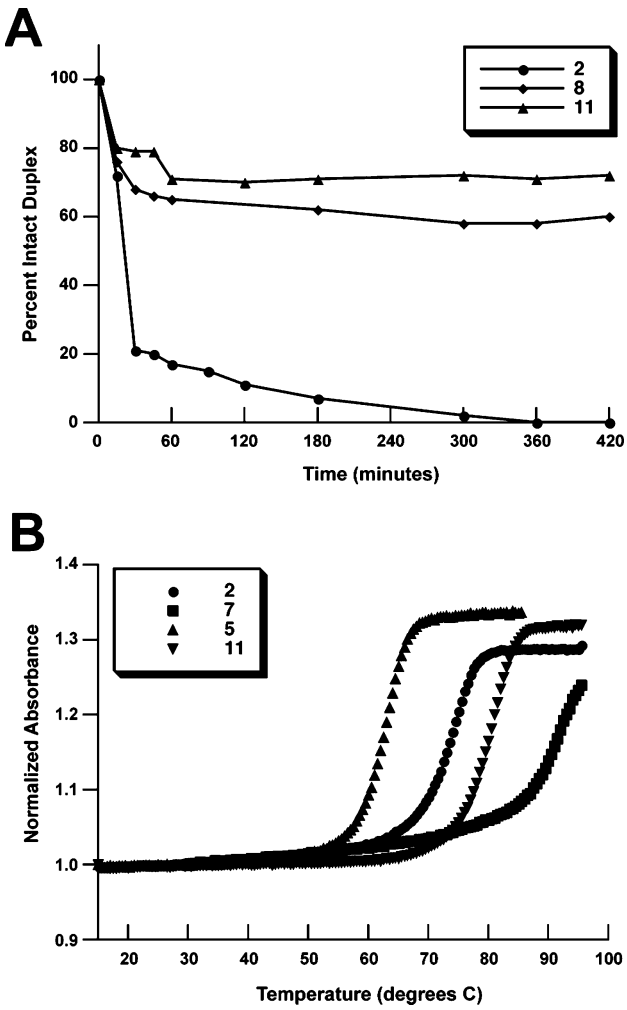

Figure 3. Comparison of the biophysical properties of unmodified blunt-ended siRNAs and 2′-F/2′-OMe modified duplexes. (A) Stability of duplexes in 25% (v/v) mouse plasma. Duplexes were treated with 25% mouse plasma at 37 °C for the indicated times, then examined by capillary gel electrophoresis to determine the amount of intact duplex (see Supporting Information for details). The plots shown are the result of two independent experiments. (B) Thermal denaturation profiles of both unmodified and modified duplexes in 100 mM NaCl, 10 mM sodium phosphate, pH 7.5, 0.1 mM EDTA, and 4 μM of each strand. Absorbance at 260 nm was measured as the temperature was raised from 15 to 85 °C or from 30 to 95 °C. Absorbances were normalized to facilitate graphical depiction. Melting temperatures (T_m) were calculated from first-derivative curves of at least two separate experiments and are summarized in Table 1.

helicase cannot overcome, shifting the bias for strand-loading more in favor of the antisense strand. We also observed that among the unmodified duplexes, the best activity comes from the duplex having the highest T_m, whereas the opposite is true for the 2′-F/2′-OMe motif. Although not definitive, these relationships may hint at the existence of an optimal thermal stability.

From these observations, the alternating 2′-F/2′-OMe motif appears to be an attractive design for creating functionally active and stable RNA duplexes. Clearly, these duplexes potently reduce levels of endogenous target mRNA, with the addition of a 5′-phosphate to the antisense strand further enhancing in vitro potency. The biophysical properties of this duplex motif, reflected in its enhanced serum and thermal stability, also favor its chances at surviving in serum, which will hopefully

translate to improved in vivo potency. The identification of this motif from our SAR analysis highlights the value of screening oligonucleotides containing multiple chemistries and may prove a useful strategy in related areas such as micro-RNA. Although promising, the utility and optimal design of this motif for in vivo applications and a biochemical explanation for the remarkable increase in potency remain to be determined and are the focus of ongoing studies. We anticipate that this and other chemically modified duplex motifs will ultimately prove useful in the design of clinically active nucleic acids.

Acknowledgment. The authors thank Bruce Ross, Mystie Nguyen, and Mingming Han for providing the 2′-*O*-methyl and 2′-deoxy-2′-fluoro nucleoside phosphoramidites, Hans Gaus, Sam Lee, and Jodee Steinberg for their assistance in oligonucleotide analysis, and Prasad Dande, Sue Freier, Bridget Lollo, and Brenda Baker for their thoughtful discussions about this project.

Supporting Information Available: Methods for the synthesis and purification of the oligonucleotides, mass spectral and capillary gel electrophoresis data of each compound, and details of the biological and biochemical assays. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Crooke, S. T. Molecular mechanisms of action of antisense drugs. *Biochim. Biophys. Acta* **1999**, *1489*, 31−44.
(2) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **2001**, *411*, 494−498.
(3) Caplen, N. J.; Parrish, S.; Imani, F.; Fire, A.; Morgan, R. A. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9742−9747.
(4) Hammond, S. M.; Bernstein, E.; Beach, D.; Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **2000**, *404*, 293−296.
(5) Martinez, J.; Patkaniowska, A.; Urlaub, H.; Luhrmann, R.; Tuschl, T. Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi. *Cell* **2002**, *110*, 563−574.
(6) Liu, J.; Carmell, M. A.; Rivas, F. V.; Marsden, C. G.; Thomson, J. M.; et al. Argonaute2 Is the Catalytic Engine of Mammalian RNAi. *Science (Washington, D.C.)* **2004**, *305*, 1437−1441.
(7) Haley, B.; Zamore, P. D. Kinetic analysis of the RNAi enzyme complex. *Nat. Struct. Mol. Biol.* **2004**, *11*, 599−606.
(8) Zhang, X.; Shan, P.; Jiang, D.; Noble, P. W.; Abraham, N. G.; et al. Small Interfering RNA Targeting Heme Oxygenase-1 Enhances Ischemia-Reperfusion-Induced Lung Apoptosis. *J. Biol. Chem.* **2004**, *279*, 10677−10684.
(9) Braasch, D. A.; Paroo, Z.; Constantinescu, A.; Ren, G.; Oz, O. K.; et al. Biodistribution of phosphodiester and phosphorothioate siRNA. *Bioorg. Med. Chem. Lett*. **2004**, *14*, 1139−1143.
(10) Song, E.; Lee, S. K.; Wang, J.; Ince, N.; Ouyang, N.; et al. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **2003**, *9*, 347−351.
(11) Zender, L.; Huetker, S.; Liedtke, C.; Tillmann, H. L.; Zender, S.; et al. Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7797−7802.
(12) Sorensen, D. R.; Leirdal, M.; Sioud, M. Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice. *J. Mol. Biol.* **2003**, *327*, 761−766.
(13) Layzer, J. M.; McCaffrey, A. P.; Tanner, A. K.; Huang, Z.; Kay, M. A.; et al. In vivo activity of nuclease-resistant siRNAs. *RNA* **2004**, *10*, 766−771.
(14) Soutschek, J.; Akinc, A.; Bramlage, B.; Charisse, K.; Constien, R.; et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature (London)* **2004**, *432*, 173−178.
(15) Chiu, Y. L.; Rana, T. M. siRNA function in RNAi: A chemical modification analysis. *RNA* **2003**, *9*, 1034−1048.
(16) Braasch, D. A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; et al. RNA Interference in Mammalian Cells by Chemically-Modified RNA. *Biochemistry* **2003**, *42*, 7967−7975.
(17) Capodici, J.; Kariko, K.; Weissman, D. Inhibition of HIV-1 Infection by Small Interfering RNA-Mediated RNA Interference. *J. Immunol*. **2002**, *169*, 5196−5201.
(18) Harborth, J.; Elbashir, S. M.; Vandenburgh, K.; Manninga, H.; Scaringe, S. A.; et al. Sequence, Chemical, and Structural Variation of Small Interfering RNAs and Short Hairpin RNAs and the Effect on Mammalian Gene Silencing. *Antisense Nucleic Acid Drug Dev.* **2003**, *13*, 83−105.
(19) Amarzguioui, M.; Holen, T.; Babaie, E.; Prydz, H. Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* **2003**, *31*, 589−595.
(20) Holen, T.; Amarzguioui, M.; Babaie, E.; Prydz, H. Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. *Nucleic Acids Res.* **2003**, *31*, 2401−2407.
(21) Czauderna, F.; Fechtner, M.; Dames, S.; Aygun, H.; Klippel, A.; et al. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* **2003**, *31*, 2705−2716.
(22) Vickers, T. A.; Koo, S.; Bennett, C. F.; Crooke, S. T.; Dean, N. M.; et al. Efficient Reduction of Target RNAs by Small Interfering RNA and RNase H-Dependent Antisense Agents. *J. Biol. Chem.* **2003**, *278*, 7108−7118.
(23) Hashimoto, J. G.; Beadles-bohling, A. S.; Wiren, K. M. Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis. *BioTechniques* **2004**, *36*, 54, 56, 58−60.
(24) Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. *Nature (London)* **2003**, *426*, 465−469.
(25) Song, J.-J.; Liu, J.; Tolia, N. H.; Schneiderman, J.; Smith, S. K.; et al. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat. Struct. Biol.* **2003**, *10*, 1026−1032.
(26) Yan, K. S.; Yan, S.; Farooq, A.; Han, A.; Zeng, L.; et al. Structure and conserved RNA binding of the PAZ domain. *Nature (London)* **2003**, *426*, 469−474.
(27) Ma, J.-B.; Ye, K.; Patel, D. J. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature (London)* **2004**, *429*, 318−322.
(28) Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. Nucleic acid 3′-end recognition by the Argonaute2 PAZ domain. *Nat. Struct. Mol. Biol.* **2004**, *11*, 576−577.
(29) Schwarz, D. S.; Hutvágner, G.; Haley, B.; Zamore, P. D. Evidence That siRNAs Function as Guides, Not Primers, in the Drosophila and Human RNAi Pathways. *Mol. Cell* **2002**, *10*, 537−548.
(30) Nykanen, A.; Haley, B.; Zamore, P. D. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell (Cambridge, Mass.)* **2001**, *107*, 309−321.
(31) Leeds, J. M.; Graham, M. J.; Truong, L.; Cummins, L. L. Quantitation of phosphorothioate oligonucleotides in human plasma. *Anal. Biochem.* **1996**, *35*, 36−43.
(32) Geary, R. S.; Matson, J.; Levin, A. A. A nonradioisotope biomedical assay for intact oligonucleotide and its chain-shortened metabolites used for determination of exposure and elimination half-life of antisense drugs in tissue. *Anal. Biochem.* **1999**, *274*, 241−248.
(33) Kawasaki, A. M.; Casper, M. D.; Freier, S. M.; Lesnik, E. A.; Zounes, M. C.; et al. Uniformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J. Med. Chem.* **1993**, *36*, 831−841.
(34) Schwarz, D. S.; Hutvagner, G.; Du, T.; Xu, Z.; Aronin, N.; et al. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **2003**, *115*, 199−208.
(35) Khvorova, A.; Reynolds, A.; Jayasena, S. D. Functional siRNAs and miRNAs exhibit strand bias. *Cell* **2003**, *115*, 209−216.

JM049167J