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Promiscuous 8-Alkoxyadenosines in the Guide Strand of an SiRNA: Modulation of Silencing Efficacy and Off-Pathway Protein Binding

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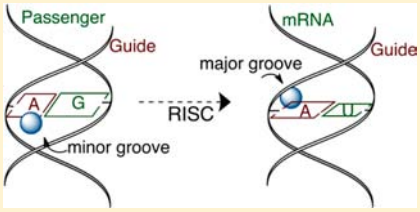
Supporting Information

ABSTRACT: 8-Alkoxyadenosines have the potential to exist in anti or syn conformations around the glycosidic bond when paired opposite to U or G in the complementary strands, thereby placing the sterically demanding 8-alkoxy groups in the major or minor groove, respectively, of duplex RNA. These modified bases were used as "base switches" in the guide strands of an siRNA to prevent off-pathway protein binding during delivery via placement of the alkoxy group in the minor groove, while maintaining significant RNAi efficacy by orienting the alkoxy group in the major groove. 8-Alkoxyadenosine phosphoramidites were synthesized and incorporated into the guide strand of caspase 2 siRNA at four different positions: two in the seed region, one at the cleavage junction, and another nearer to the 3′-end of the guide strand. Thermal stabilities of the corresponding siRNA duplexes showed that U is preferred over G as the base-pairing partner in the complementary strand. When compared to the unmodified positive control siRNAs, singly modified siRNAs knocked down the target mRNA efficiently and with little or no loss of efficacy. Doubly modified siRNAs were found to be less effective and lose their efficacy at low nanomolar concentrations. SiRNAs singly modified at positions 6 and 10 of the guide strand were found to be effective in blocking binding to the RNA-dependent protein kinase PKR, a cytoplasmic dsRNA-binding protein implicated in sequence-independent off-target effects.



INTRODUCTION

Chemical modification of small interfering RNAs (siRNAs) is necessary to achieve potent RNA interference (RNAi) therapeutics. Modifications of the ribose moiety[1−18] and phosphodiester backbone[1,2,7] have improved siRNA stability,[1,9] ribonuclease resistance,[1,3,6,8,12] potency,[3,8,17] and specificity.[1,19] Polymer,[20−22] lipid,[23] cholesterol,[24−26] carbohydrate,[20,21] peptide,[27] small molecule[24] terminal modifications, and delivery systems of siRNAs exhibit effective RNAi, and polymer- and cholesterol-based conjugates facilitated intracellular delivery of siRNA both in vitro and in vivo. Although more challenging, a few successful siRNA base modifications have also been reported.[2,28−34] SiRNA base modifications[35] are relatively less explored as compared to sugar modifications or terminal modifications, because preservation of necessary hydrogen-bonding interactions and the stable A-form duplex is crucial in effective RNAi. Disruption of hydrogen bonding (e.g., in N^3-Me-U) or steric occlusion of siRNA-RISC interactions (5-IU) can adversely affect the RNAi efficacy and specificity.[2] For this reason, most of the successful base modifications have been introduced into either passenger strands or at the 3′-end of guide strands.[28,32,34]

Base modifications have been shown to enhance thermal stability[31] and nuclease stability;[30] these also helped visualize intracellular trafficking[34] and reduced off-target effects.[28,32,33] Kool and co-workers, and Manoharan, Egli, and co-workers, reported substitution of pyrimidines (uridine) in the guide strands of siRNAs with more hydrophobic aromatic ring systems (e.g., 2,4-difluorobenzene[29] and 2,4-difluorotoluene[30,36]), although here silencing efficacy varied depending on the position of the substitution. Recently, major groove substitution of modified purines in various positions of the guide strand has been reported; here, also RNAi efficacy was found to be position dependent.[37]

Along with the targeted delivery issue, off-target effects are still one of the major limitations for specific and selective action of therapeutic siRNA in vivo. Off-target effects leading to immunostimulation pose a significant limitation on the therapeutic use of siRNA. Specific siRNA sequences can bind to toll-like receptors and lead to immunostimulation.[38−42] In addition, sequence-independent off-target effects involve binding of virtually any siRNA with double-stranded RNA binding motif (dsRBM) containing proteins, for example, RNA-dependent protein kinase (PKR), adenosine deaminases (ADARs), and other intracellular proteins.[43,44] PKR activation leads to phosphorylation of eIF2α, inhibition of translation initiation, and other antiviral signaling events.[45] Although siRNAs are shorter than ligands that trigger high PKR activity, lower level activation of PKR has been observed with siRNAs in vitro and when transfected into certain cell types.[38] ADARs also bind dsRNA and have been shown to interact with substrates within the RNAi pathway.[46,47] The full-length isoform of ADAR1 (ADAR1p150) has been linked to decreased siRNA potency in mammalian cells, presumably due to the formation of high affinity siRNA·ADAR1p150 complexes within the

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cytoplasm.[44] Additionally, ADAR has been shown to reduce RNAi efficiency in *Drosophila* cell culture.[47] Therefore, prevention of siRNA binding to dsRBM proteins off the RNAi pathway is important for potency and specificity of therapeutic RNAi. Some nucleoside analogues[48] have shown promise in preventing PKR binding, but a complete study with modified sugars to address such sequence-independent off-target effect is not available. On the other hand, altering the RNA minor groove through base modifications has shown potential in modulating siRNA properties and siRNA–protein interactions. The Beal laboratory initiated a systematic study on siRNA base modifications that could block off-pathway protein binding,[35] and they have also shown that pendent minor groove modifications of guanines[28] and 2-aminopurines[32] in the passenger caspase 2 siRNA can successfully prevent siRNA–dsRBM interactions, while maintaining siRNA efficacy.

Base modifications in the guide strand are more challenging, because many modifications at crucial siRNA sites (e.g., in the middle of the seed region or at the cleavage site) will drastically reduce the silencing efficacy or might even completely abolish the knockdown of the desired mRNA. In contrast, modifications at those sites might, in fact, turn out to be more exciting and lead to elucidation of crucial mechanisms in the RISC complex. For example, appropriate modifications in the minor or major groove of an siRNA can help explore the siRNA mechanisms in detail, and placement of designer modifications in those grooves can provide further novel insights into the siRNA–RISC interactions. Our laboratories have previously shown that N^2-alkylated 8-oxo-7,8-dihydro-2′-deoxyguanosine-containing siRNAs can substantially prevent siRNA–PKR interaction by placing a "switchable" alkyl group in the minor groove of an siRNA during delivery; in the RISC, the modified siRNAs can flip the sterically encumbering group into the major groove and maintain good RNAi efficacy.[33] However, 8-oxoguanine-containing DNA oligomers are known to be inflammatory and immunostimulatory themselves.[49,50] Therefore, we chose to explore alternative purine modifications in the guide strand. Here, we report the synthesis, thermal stability, and biological activity of 8-alkoxyadenosine-substituted siRNAs.

8-AlkoxyA phosphoramidites were synthesized and incorporated into the guide strand of a siRNA targeting the caspase 2 mRNA sequence. Modified siRNAs were tested for their RNAi efficacy and ability to address off-target effects due to siRNA–PKR interactions. 8-Substituted adenosines and guanosines have been shown to exist in an equilibrium mixture of syn/anti conformers. In this work, 8-alkoxyadenosines are postulated to flip between anti and syn conformations depending on the base-pairing partner. In the natural anti conformation, 8-alkoxyA will base pair with U, whereas, in the syn conformation, the Hoogsteen face of the nucleoside will be exposed for base pairing, and its best complement would be anti G. We propose that during delivery of the siRNA, 8-alkoxyA in the guide strand (opposite to G in the passenger strand) would project its steric blockade into the minor groove of siRNA, thereby preventing intracellular protein binding onto the RNA. When the siRNA is recruited into the RISC assembly, the 8-alkoxyA in the guide siRNA would encounter U in the mRNA and would flip the bulky appendage into the major groove, thereby allowing necessary guide strand–mRNA–RISC assembly to form (Figure 1).

Alkyl groups were chosen on the basis of their size and shape: propargyl, phenethyl, and cyclohexylethyl. The rationale

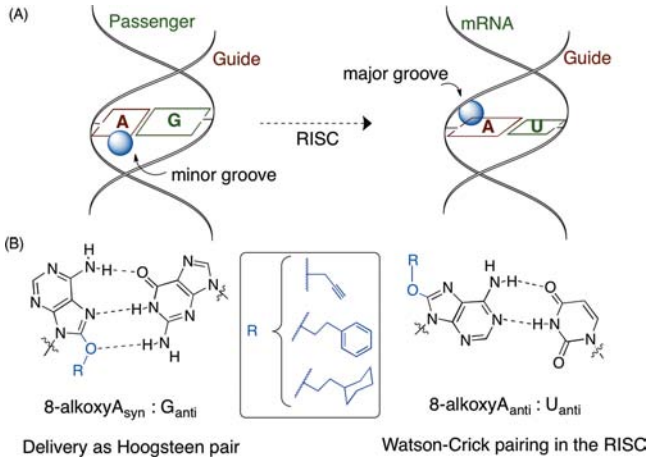

Figure 1. (A) Proposed "base switch" showing flipping of a steric blockade from the minor to the major groove; steric occlusion in the minor groove prevents siRNA–PKR interaction in transit, and major groove accommodation of the steric blockade allows siRNA–protein interactions in the RISC. (B) Proposed base pairs of the 8-alkoxyAs.

behind the choices is that smaller groups (propargyl) might exhibit higher duplex stabilities and better mRNA knock down efficiencies, whereas a larger group (cyclohexylethyl) might prevent immunostimulation to a greater extent, and a medium sized group (phenethyl) might serve both purposes equally efficiently. Our study suggests that the mRNA knock down ability of the singly modified siRNAs is quite similar to that of the unmodified positive control siRNA. Singly modified siRNA oligomers have also exhibited reduced tendency to bind PKR. With additional alkoxy substitution, the efficiency of the siRNA is significantly reduced; therefore, multiply modified siRNAs were not tested for off-pathway protein interactions.

RESULTS AND DISCUSSION

Design and Synthesis of 8-Alkoxyadenosine Phosphoramidites. 8-AlkoxyA phosphoramidites were synthesized in multiple steps from adenosine (Figure 2). Detailed step-by-step syntheses can be found in the Supporting Information. Adenosine was first brominated at C8 following a standard procedure,[51] and sugar hydroxyl groups were protected by silylating agents. 5′-OH and 3′-OH groups were protected using a bidentate silylating agent,[52] and the remaining 2′-OH was protected using a TBDMS protecting group. Next, the bromide ion was displaced with an alkoxy groups via an aromatic nucleophilic substitution reaction. The alkoxide anion was generated in situ by adding dropwise nBuLi into the corresponding alcohol in THF. The exocyclic amine of adenosine was protected using a benzoyl group. Next, the 5′- and 3′- hydroxyls were deprotected using HF–pyridine, a mild defluorinating reagent, leaving 2′-OH protection intact. The 5′-OH was then protected with a standard DMT group, and the 2′-OH was converted to a phosphoramidite derivative.

Propargyl, phenethyl, and cyclohexylethyl groups were chosen on the basis of the size and shape of the alkyl groups. The choice of alkyl groups was also considered in terms of prevention of siRNA–PKR interaction as well as maintenance of target mRNA knockdown. We thought that methyl or ethyl groups might be too small to have any significant impact on the siRNA–protein interactions. However, it was also noted that too large a group in the guide strand might reduce significantly or even completely abolish the RNAi efficacy. Hence, we

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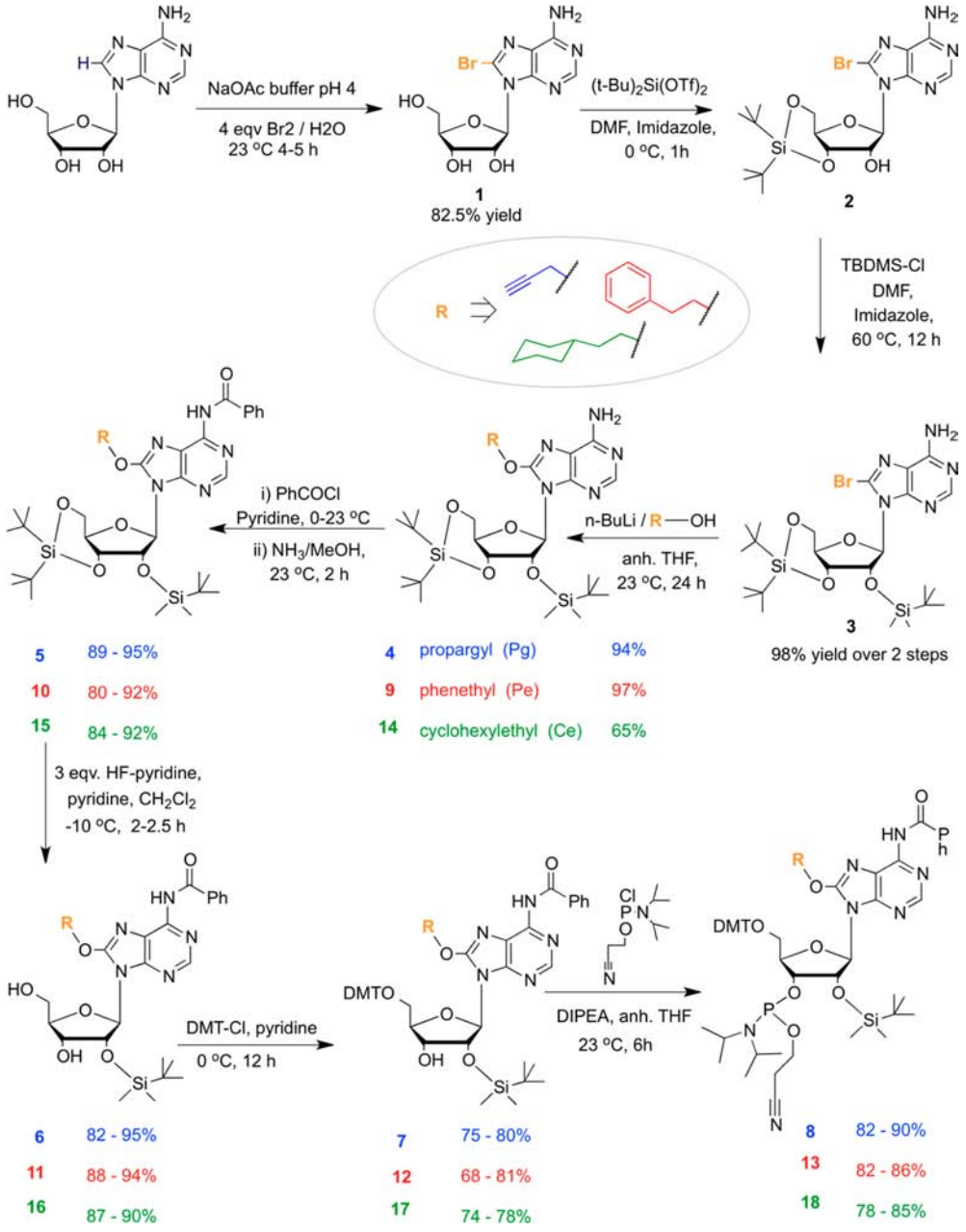

Figure 2. Synthesis of the 8-alkoxyadenosine phosphoramidites.

started the series from the propargyloxy modification, which was expected to retain the RNAi efficacy, as well as prohibit the unwanted protein binding to some extent. The phenylethoxy group was chosen as the most promising group in the guide strand, which could serve both purposes. We were optimistic that cyclohexylethyl modifications will prevent dsRBM-containing proteins from binding siRNAs, but were interested to explore whether such a large modification might also compromise RNAi efficacy.

Design of the Caspase 2 SiRNA and Synthesis of Modified Guide Strands. Caspase 2 siRNA (A:U) and the corresponding negative control siRNA as a scrambled sequence were designed using Ambion's siRNA designing tool and checked for sequence similarity using nucleotide Basic Local Alignment Search Tool (BLAST). No significant similarity was found with other genes. To analyze caspase 2 expression levels quickly and reliably, a plasmid-based dual luciferase assay system (psiCHECK2 vector) (Supporting Information Figure S38) was employed. A fragment of the caspase 2 mRNA sequence (Supporting Information Figure S39) was inserted into the vector, and the resulting reporter plasmid was used to evaluate caspase 2 mRNA knockdown. The siRNAs successfully knocked down caspase 2 mRNA in cultured HeLa cells. Using standard solid-phase RNA synthesis, 8-alkoxyA phosphoramidites were effectively coupled into the guide strand to afford four singly modified and three doubly modified oligonucleotides.

Thermal Analysis of dsRNAs with 8-AlkoxyAs. Unmodified and modified (8-alkoxyA-containing) guide strands were annealed to the complementary passenger strands (Figure 3). The guide and passenger strands were designed in such a way that 8-alkoxyA faces G or U in the corresponding passenger strands. The unmodified A:U duplex was used as a reference, based upon which the thermal stabilities of the singly

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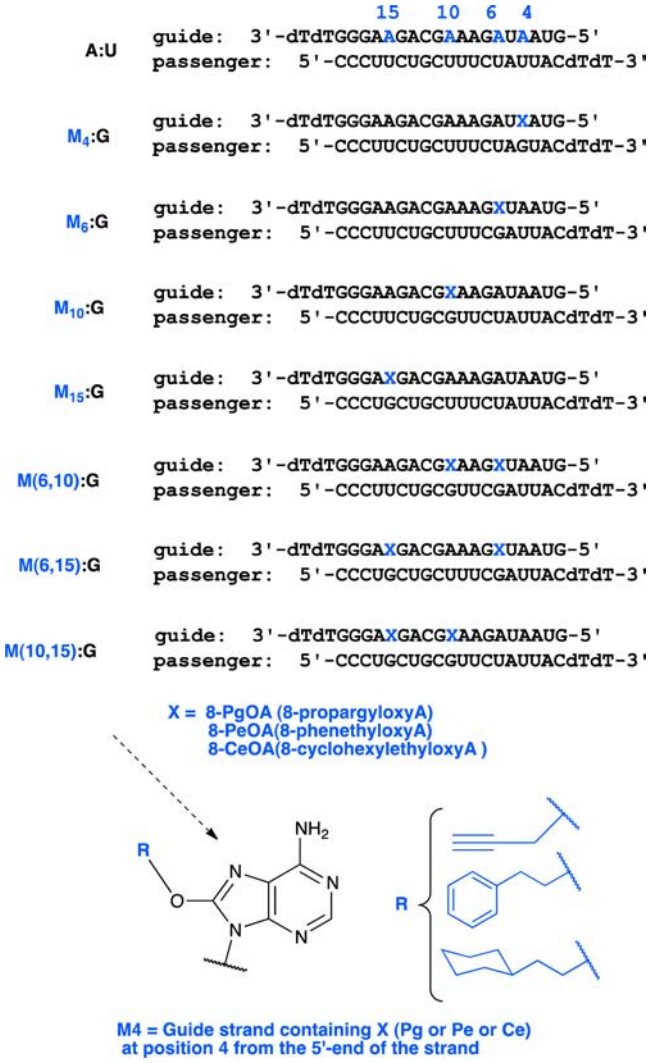

Figure 3. SiRNA sequences used. A:U represents unmodified siRNA, and modified (denoted by M:G) sequences have the standard A:U base pair replaced by 8-alkoxyA(X):G in one or two positions in the siRNAs.

and doubly modified duplexes (both 8-ROA:U and 8-ROA:G) were analyzed. In the case of the 8-ROA:U base pair, the 8-ROA nucleoside can adopt the canonical anti orientation around the glycosidic bond. For the 8-ROA:G pair, 8-ROA_{syn}:G_{anti} and 8-ROA_{anti}:G_{anti} combinations were considered, while other A:G combinations were deemed too distorting to exist in an A-form duplex RNA. However, the 8-ROA_{anti}:G_{anti} pair will widen the helix diameter considerably and introduce strain into the duplex; additionally, the presence of a significantly large alkoxy group at position 8 of the purine ring favors the 8-ROA_{syn}:G_{anti} combination. Thus, the existence of 8-ROA_{anti}:G_{anti} cannot be ruled out, but 8-ROA_{syn}:G_{anti} is more likely to occur in A-form duplex RNA.

All of the singly and doubly modified duplexes displayed lower melting temperatures (T_m) as compared to unmodified duplexes (Figures 4 and 5). Therefore, the alkoxy group appears to introduce instability in the RNA duplexes. In all duplexes, the 8-ROA:U base pair was found to be more stable than the 8-ROA:G base combination, implying that the Watson−Crick hydrogen bonding is still preferred in these modified adenosines, provided that 8-ROA faces U as the complementary base. Although 8-ROA:G also has a greater potential to expose its Hoogsteen face to anti G, steric clash between the alkoxy group of 8-ROA and the exocyclic amine of G in the minor groove might undermine the strength of hydrogen bonding, resulting in lower T_m values for 8-ROA:G.

Melting temperatures also varied depending on the position of the 8-alkoxyA base in the guide strand. It is not clear why the 5′-end of the guide strand can tolerate modifications of variable size more effectively than the other end. The 5′-end of the guide strand is thermodynamically less stable and hence more prone to unzipping; introducing a destabilizing nucleoside in this region, as opposed to the other end, should further decrease the thermal stability of the duplex. Again at positions 10 and 15, the 8-alkoxyAs are between two purines, so a greater stacking interaction is expected. These siRNAs would be expected to have higher melting temperatures as compared to siRNAs bearing these modifications at positions 4 and 6. In practice, however, the reverse trend is observed. In all cases, higher duplex stability was obtained when the modifications are closer to the 5′-end of the guide strand. Modifications in the middle position (10) or toward the 3′-end (15) of the guide strand always exhibited reduced thermal stability.

For single modifications, lowering of the T_m was moderate for modifications at positions 4 and 6 opposite U (e.g., Pg4:U, Pg6:U, Ce4:U); however, single modifications at positions 10

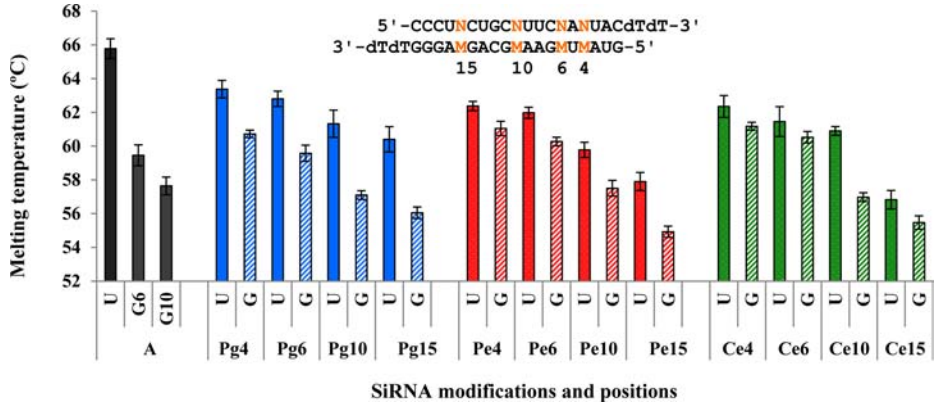

Figure 4. Thermal analysis of siRNA duplexes with single 8-alkoxyA modifications at four different positions, 4, 6, 10, and 15, in the guide strand. Modifications were placed opposite to U or G in the corresponding passenger strands. The sequences are shown in Figure 3; here, X represents 8-PgOA, 8-PeOA, or 8-CeOA, and N is either U or G.

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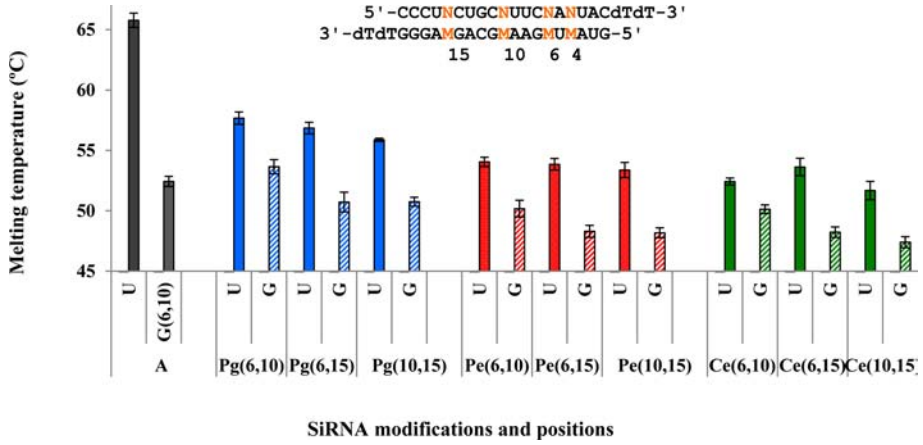

Figure 5. Thermal analysis of siRNA duplexes with double 8-alkoxyA modifications at three different positions, 6, 10, and 15, in the guide strand. Modifications were placed opposite to U or G in the corresponding passenger strands. The sequences are shown in Figure 3; here, X is 8-PgOA, 8-PeOA, or 8-CeOA, and N is either U or G.

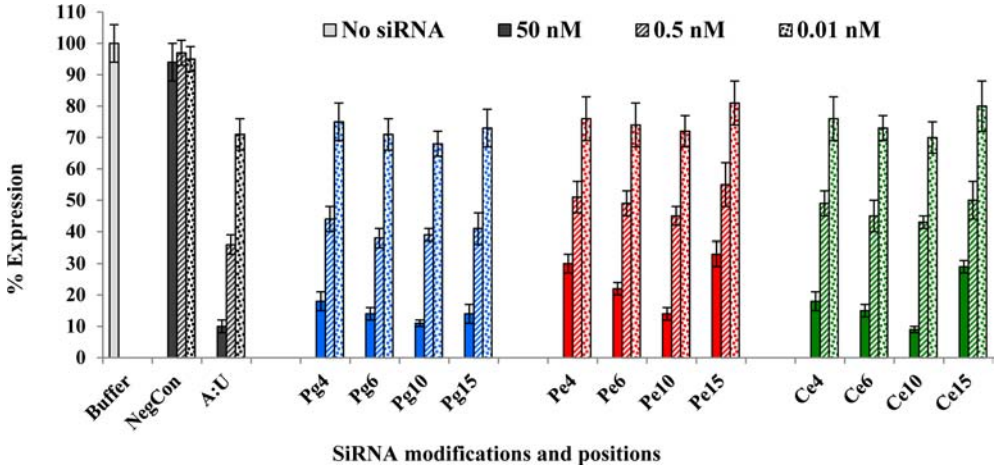

Figure 6. % Expression of Renilla luciferase relative to firefly luciferase when treated with siRNAs bearing single 8-alkoxyA modification at position 4, 6, 10, or 15 in the guide strand. 8-AlkoxyA modifications were placed opposite G (in the passenger strand) during delivery and targeted U (in the mRNA) in the RISC. The siRNA sequences are depicted in Figure 3. Experiments were conducted at three different concentrations: 50, 0.5, and 0.01 nM.

and 15 exhibited significantly lower T_m irrespective of their placement opposite to G or U. As expected, reduction in duplex stability was more pronounced (7−14 °C) for doubly modified duplexes. Interestingly, except for position 15, the size of the alkoxy groups did not have much influence on the thermal stability of siRNA duplexes.

A similar trend was noticed in 8-ClA-containing RNA duplexes, which also have lower melting temperatures as compared to the unmodified duplexes.[53] Therefore, thermal destabilization cannot be primarily linked to the substituent size and shape effect, but also to substituent electronegativity values. Because both Cl and O have higher electronegativities as compared to H, a strong electron-withdrawing inductive effect may operate to reduce electron density in the purine rings significantly, and hence this can reduce the stability of hydrogen bonds formed by 8-ClA:U or 8-ROA:U in the Watson−Crick base pairing and affect base stacking as well.

Lowering of the T_m in the 8ROA:U base pair as compared to the A:U base pair can also be explained in terms of a relatively pronounced *syn* preference of 8-substituted purines over the anti conformation, both in the context of DNA[54,55] and RNA[53,56−58] nucleosides and duplex oligonucleotides. X-ray crystallographic[56] and CD studies[57] of 8BrA and the corresponding 5′-monophosphate have shown that these molecules predominantly exist in the syn conformation around the glycosidic bond due to the presence of a sterically demanding Br atom at position 8 in the purine ring. Although the van der Waals radius of Cl (1.80 Å) is significantly smaller than that of Br (1.95 Å), theoretical calculations predicted preferentially the syn conformation even for 8-ClA nucleosides.[58] Importantly, a relative lowering of melting temperature of A:U versus A:G combination was noticeably greater than the 8-ROA:U versus 8ROA:G combinations. Interestingly, this difference shrank even more for the 8-PeOA- and 8-CeOA-containing duplexes, implying that with increasing the steric bulk of the alkoxy group, the syn preference of the 8-alkoxyadenosines increased. All of these observations fit well with the preferential syn conformation of the 8-substituted-adenosines, such as 8-ClA and 8-ROA, in the duplex RNA context.

Caspase 2 mRNA Knockdown Studies with Modified Adenosine-Containing SiRNAs. Singly substituted siRNAs, alkoxylated at several positions of the guide strand, exhibited almost equivalent or slightly lower silencing effects as compared

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to unmodified caspase 2 siRNAs (Figure 6). Among singly modified siRNAs, 8-PgOA exhibited the highest mRNA knockdown efficiency. Surprisingly, 8-CeOA modifications, even though the largest among three modifications, showed almost equal knockdown efficiency to 8-PgOA, whereas 8-PeOA containing siRNAs were found to have the lowest efficacy. Thus, the mRNA knockdown ability of the modified caspase 2 siRNAs does not directly correlate with the substituent size.

Interestingly, singly modified siRNAs with intermediate thermal stability (modifications at positions 6 and 10) exhibited higher RNAi efficacy than those having the highest (modification at position 4) or the lowest (modification at position 15) stability. This observation supports the basic rules of siRNA design and efficacy, which emphasizes that, for optimal efficacy, the thermal stability of the siRNA duplexes be intermediate, neither very high, nor very low.[59,60] Because the differences in T_m among the four sequence contexts are not very high, their influence on the RNAi efficacy engenders curiosity.

In all cases, modifications at position 10 of the guide strand exhibited the highest gene-silencing efficacy, followed by position 6. Modifications at position 15 seemed to reduce the potency of siRNAs, with the exception of the propargyloxy modification, which was found to be equally active when compared to other positions in the series. So, position 15 was found to tolerate smaller groups better as opposed to larger groups such as phenylethoxy and cyclohexylethoxy groups. This trend is somewhat unexpected, because it is already known that substitutions toward the 3′-end of the guide strand typically can tolerate chemical modifications and even mismatches rather well.[32,34] Contrarily, the seed region and the cleavage site are more sensitive to chemical modifications and mismatches, in general. Sometimes, a single mismatch[59] at the center or chemical modifications distorting the A-form duplex structure[2] abolishes the efficacy of the corresponding siRNA. In this case, modifications in two important positions of the seed region with large substituents are quite well tolerated. More surprisingly, a large modification adjacent the cleavage site (position10) actually rendered the siRNA more effective toward knocking down caspase 2 mRNA. This trend was observed for all modifications; however, with larger modifications such as 8CeOA and 8PeOA, this trend was observed without any ambiguity. The presence of a hydrophobic pocket at the corresponding position in Argonaute2 in the RISC might be a plausible explanation for this observation.

Multiple 8-ROA substitutions at the guide strands showed significantly reduced silencing efficacy as compared to their corresponding positive control siRNAs or single modifications (Figure 7). This trend holds irrespective of the substituent size, shape, and position. These siRNAs only exhibit significant mRNA knock down at 50 nM concentrations. Modified purines at positions 6 and 10 in the guide strand opposite to G or U (in the passenger strand) did not substantially change the knock down efficiency (Figure 8). In most of the cases, delivering the modified purine opposite to G brought about almost equal or slightly higher mRNA knockdown than delivering it opposite to U. Only with Ce6 was a reverse trend observed, although it was only by a small margin. These data suggest that both 8-ROA:G and 8-ROA:U can be loaded into the RISC with equal efficiency.

Importance of Switching the Steric Blockade from the Minor to the Major Groove in the RISC. To explore the importance of "base switching" in the context of RISC-mediated cleavage of caspase 2 mRNA, two mutant plasmids, P6 and P10, were synthesized using appropriate inserts by mutating specific positions of the wild-type caspase 2 insert. Two important sites in the guide strand of the caspase 2 siRNA, position 6 in the seed region and position 10 adjacent the cleavage site, were chosen for the construction of mutant plasmids, P6 and P10. In such cases, the 8-alkoxyAs (in the guide strand) face G as the complementary base both during their delivery opposite to the passenger strands and during interaction with the mRNA in the RISC. Therefore, alkoxy steric blockades will always be presented in the minor groove both in the guide−passenger and in the guide−mRNA constructs.

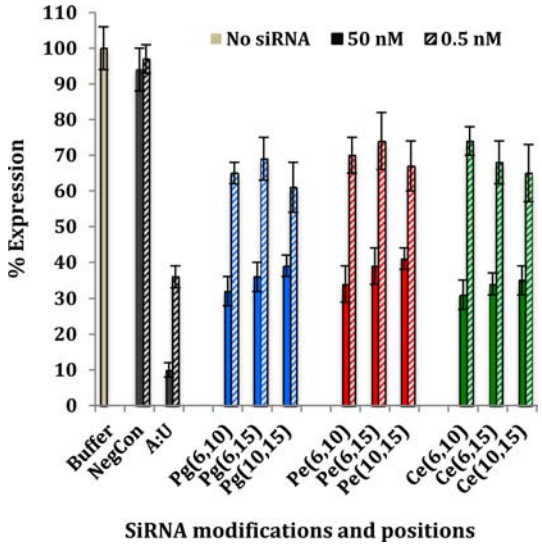

Figure 7. % Expression of the Renilla luciferase relative to the firefly luciferase when treated with siRNAs bearing double 8-alkoxyA modifications in three different combinations in the guide strand: (6, 10), (6, 15), and (10, 15). 8-AlkoxyA modifications were placed opposite to G in the passenger strand during delivery and targeted U (in mRNA) in the RISC. The siRNA sequences are depicted in Figure 3. Experiments were conducted at two different concentrations: 50 and 0.5 nM.

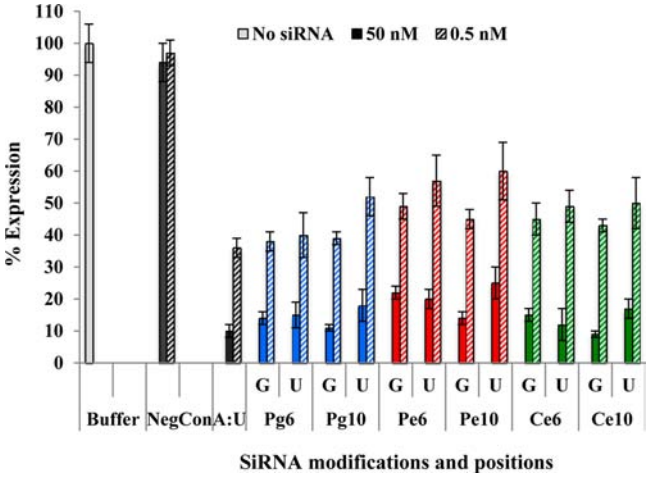

Figure 8. % Expression of the Renilla luciferase relative to the firefly luciferase when treated with siRNAs bearing 8-alkoxyA modifications at positions 6 and 10 opposite to either G or U.

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As expected, the mRNA knockdown efficiency was drastically reduced for all three (8-PgOA, 8-PeOA, and 8-CeOA) modifications; 8-PgOA being the smallest in size is relatively more efficacious (Figures 9 and 10). In the case of A:G mismatched guide SiRNA:mRNA constructs, a similar decrease in mRNA knockdown efficiency was noticed with respect to both mutant plasmids, P6 and P10.

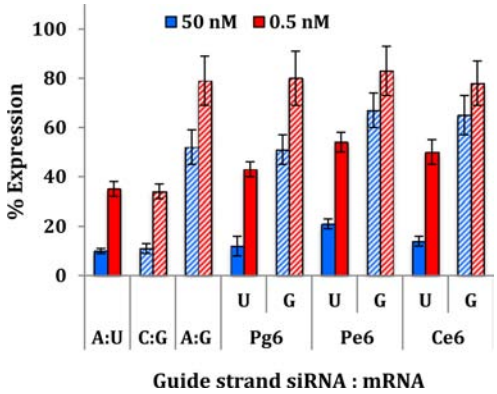

Figure 9. % Expression of the Renilla luciferase relative to the firefly luciferase, when treated with siRNAs bearing modifications at position 6 in the guide strand, and targeted U (from dA6:dT plasmid, depicted by solid bars) versus G (from dC6:dG plasmid, depicted by hashed bars) in the caspase 2 insert and mutant caspase 2 insert mRNA, respectively. Sequences are given in Figure 2. Experiments were conducted at two different concentrations: 50 and 0.5 nM. A:U, Pg6:U, Ce6:U, and Pe6:U represent targeting of U in the caspase 2 insert mRNA (from dA6:dT plasmid), and C:G, A:G, Pg6:G, Ce6:G, and Pe6:G represent targeting of G in the mutant mRNA where a U was mutated to G (from dC6:dG plasmid). Nomenclature of the plasmid comes from the corresponding modification position in the guide strand of caspase 2 insert mRNA.

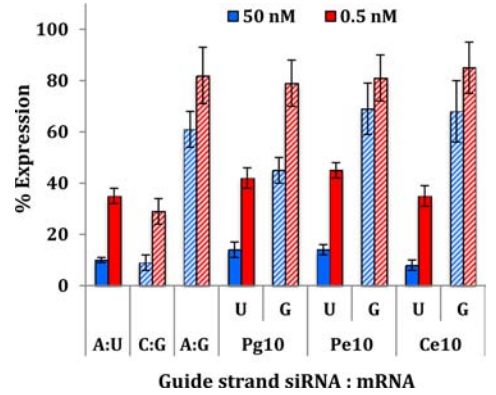

Figure 10. % Expression of the Renilla luciferase relative to the firefly luciferase, when treated with siRNAs bearing modifications at position 10 in the guide strand, and targeted U (from dA10:dT plasmid, depicted by solid bars) versus G (from dC10:dG plasmid, depicted by hashed bars) in the caspase 2 insert and mutant caspase 2 insert mRNA, respectively. Sequences are given in Figure 2. Experiments were conducted at two different concentrations: 50 and 0.5 nM. A:U, Pg10:U, Ce10:U, and Pe10:U represent targeting of U in the caspase 2 insert mRNA (from dA10:dT plasmid), and C:G, A10:G, Pg10:G, Ce10:G, and Pe10:G represent targeting of G in the mutant mRNA where a U was mutated to G (from dC10:dG plasmid). Nomenclature of the plasmid comes from the corresponding modification position in the guide strand of caspase 2 insert mRNA.

These results indicate that for the guide:mRNA duplex, both local widening[36] (due to the A:G pair) and minor groove crowding (due to the 8ROA:G pair) can lead to drastic reduction of the mRNA knockdown efficacy. Probably these distortions in the guide:mRNA duplex do not allow the necessary protein side-chain interactions in the guide:mRNA:R-ISC ternary complex.

SiRNA–PKR Binding Studies. To assess the effect of these modifications on siRNA binding to an off-pathway dsRNA-binding protein, we used a PKR binding assay involving immobilized biotinylated siRNAs as previously described.[32] 5′-Biotinylated passenger strands with either U or G opposite to the modified purines were annealed to Pg6, Ce6, Pe6, Pg10, Ce10, Pe10, and unmodified guide strands. The biotinylated strands were then attached to magnetic streptavidin beads and used in affinity purification for PKR. The degree of PKR binding for each strand was then analyzed by Western blot.

Our results show that different types of modifications, when placed in the guide strand opposite to G or U in the passenger strand, are significantly less prone to bind to PKR as compared to the unmodified sequences (Figure 11). Again, unmodified

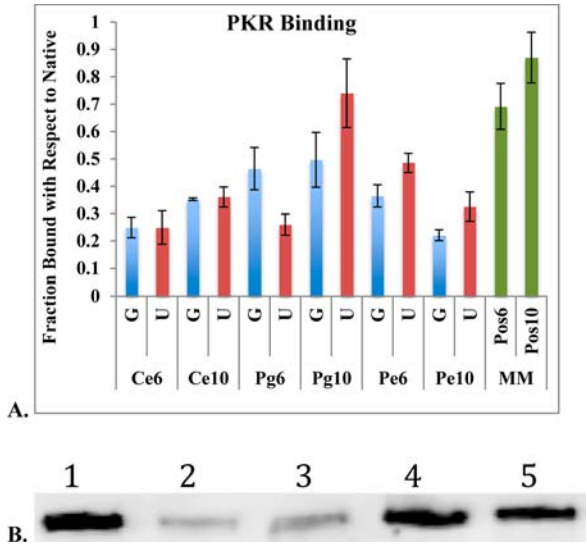

Figure 11. PKR binding to modified siRNAs containing 8-alkoxyadenosine switches. (A) Biotinylated siRNAs were bound to magnetic streptavidin beads and treated with lysates from U87 cells treated with IFN-α. The amount of PKR retained was determined by Western blotting. Cyclohexylethoxy (Ce), phenylethoxy (Pe), and propargyloxy (Pg) modifications were tested at positions 6 and 10 opposite either G (blue) or U (Rred). Strands with A:G mismatches were also tested at positions 6 and 10 as controls (green). (B) A representative Western blot consisting of (1) unmodified siRNA, (2) PgG6, (3) PgU6, and (4,5) Pos6 in duplicate.

siRNAs with A:G mismatches at positions 6 and 10 are almost as susceptible to bind PKRs as the unmodified one. Hence, prevention of protein binding is not a result of mere bulges due to A:G mismatches, but rather an outcome of placement of different types of steric blockades in the minor and major grooves by means of placing 8-alkoxyA opposite to U or G.

With propargyloxy modifications being the smallest in size of those studied here, Pg6:G, Pg10:U, and Pg10:G were the least effective in preventing PKR binding with the exception of Pg6:U, whereas all cyclohexylethoxy and phenylethoxy modifications reduced the PKR binding significantly. It seems

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that PKR is slightly more prone to bind to modified siRNAs where U in the passenger strand was placed opposite to the modification in the guide strand than to siRNAs where modifications are opposite to G. In some cases (for example, Pg10:U vs Pg10:G, Pe10:U vs Pe10:G), this discrimination is more pronounced, and in some cases (Pg6:U vs Pg6:G, Pe6:U vs Pe6:G) less. These data suggest that this type of steric occlusion of the minor groove is moderately or marginally advantageous over major groove occlusion. Surprisingly, when 8-CeO modifications were placed in positions 6 and 10 opposite to U or G, no distinction was observed between the binding affinities toward PKR. Both Ce6:G and Ce6:U were equally effective in preventing PKR binding. A similar trend with lesser efficacy was observed with Ce10:G and Ce10:U.

There are other reports of prevention of PKR binding by manipulating minor groove modifications;[28,32,33] however, there is no report, so far, of any major groove modification that is equally efficient in attenuating siRNA−PKR interaction when placed in the same position of an siRNA. 8-PeOA and 8-CeOA modified siRNAs are the first examples to exhibit such a characteristic. From the thermal analysis data and RNAi studies with mutant plasmids, it is clear that 8-ROA:U and 8:ROA:G combinations are distinct, their differences likely related to different glycosidic bond conformations and placement of the steric blockades. Previously, Kannan et al. reported N^2-alkylated 8-oxoguanine-based switches are capable of preventing PKR binding only when the steric blockade is placed in the minor groove as opposed to the major groove.[33] In that study, N^2-propyl- and N^2-benzyl-modified 8-oxoguanines were used. The phenylethoxy and cyclohexylethoxy groups used here are not only bulky, but also significantly longer than the propyl and benzyl groups used in the previous studies. Hence, irrespective of their position in either groove, these larger groups repel PKR to a much greater extent even when placed in the major groove.

Another probable rationale for the high activity of 8ROA:U might be the modification site (position 8) of adenosine itself. PKR is known to bind dsRNA by interacting with two segments of the minor groove and the intervening major groove.[61] In the major groove, lysine side chains of PKR interact with the negatively charged phosphate backbone of dsRNA. Hence, appropriate major groove modifications of the siRNA can potentially prevent this electrostatic interaction and block PKR binding. In the case of $8ROA_{anti}$:U-containing siRNAs, the alkoxy group is suitably positioned to disrupt such major groove interactions with the siRNA.

CONCLUSIONS

A general solution to sequence-independent off-pathway protein binding of siRNAs, while maintaining mRNA knock down efficacy, was investigated here. This study also explored the scope of guide strand modifications by utilizing 8-alkoxyadenosines of variable alkyl group size. This is an unusual example in which a purine ribonucleoside is proposed to exist in both syn and anti conformations around the glycosidic bond depending on the base-pairing partner. 8-Alkoxyadenosine modifications were tolerated both in singly and in doubly modified duplexes irrespective of G or U as the pairing partner, although lowering of the T_m is very pronounced in cases of multiple modifications. Singly modified siRNAs were almost the same or slightly less effective in RNAi, whereas multiply modified siRNAs exhibited much lower caspase 2 insert mRNA knock down activity. Singly modified siRNAs were capable of inhibiting PKR−siRNA interactions as compared to the unmodified siRNA. The necessity of "base switching" in the RISC was demonstrated by using two caspase 2 inserts, individually mutated at positions 6 and 10. To confirm the syn versus anti conformational preference of 8-substituted adenosines in appropriate duplex RNA contexts, NMR spectroscopic studies are currently in progress.

METHODS

Synthesis of 8-Alkoxyadenosine Phosphoramidites. Complete experimental details and spectra are given in the Supporting Information.

Synthesis and Purification of SiRNAs. All siRNA oligonucleotides, modified and unmodified, were synthesized by standard solid-phase RNA synthesis on DNA columns in the DNA/Peptide Core Facility of the University of Utah using an Applied Biosystems Model 394 DNA/RNA synthesizer. After synthesis, the 21-mer RNA oligonucleotides were cleaved from the column and deprotected using methanolic ammonia for 24 h at room temperature, and the 2′-OTBDMS group was deprotected by using TEA·3HF overnight at room temperature. The oligomers were then dialyzed at 4 °C for 6 h and lyophilized in a SpeedVac. SiRNA oligomers were purified by semipreparative ion-exchange HPLC using ammonium acetate and lyophilized to remove salt and water. The remaining salt was removed by dialysis. The samples were stored at −20 °C under dry conditions.

SiRNA Duplex Formation. SiRNA oligomers were dissolved in annealing buffer (100 mM NaCl and 10 mM TRIS, pH 7.1), and appropriate guide and passenger strands were mixed in equal amounts in an Eppendorf tube. The nucleotide mixtures were placed in a 90 °C water bath for 5 min, and the bath was gradually cooled to room temperature in about 2 h.

Thermal Analysis of SiRNA Duplexes. Melting temperature experiments of the hybridized duplexes were performed in a Beckman DU 650 spectrophotometer. In all experiments, the temperature was varied from 25 to 80 °C, and the rate of heating was 0.5 °C/min. Thermal denaturation was monitored at 260 nm. All experiments were performed with 1 nmol of duplex RNA in siRNA annealing buffer (containing 10 mM Tris, 100 mM NaCl, pH 7.1) in triplicate and normalized to appropriate blank controls. The reported T_m value was the average of three independent experiments, and error bars represent standard deviation from the average value. Experimental data were analyzed by "two point average" method. Four cuvettes were filled with 325 μL of annealing buffer and were read as blanks. Next, three cuvettes were emptied, and 1 nmol of siRNA duplex was added to the siRNA annealing buffer so that the total volume of the mixture became 975 μL. This mixture was equally distributed in those three cuvettes, and the fourth cuvette was loaded with 325 μL of annealing buffer. Lids were closed tightly, and the temperature was varied from 25 to 80 °C.

Synthesis of Plasmids. Appropriate caspase 2 inserts were introduced into the psiCHECK2 vector using the multiple cloning region of the plasmid. One wild-type and two mutant plasmids were synthesized. The plasmids were multiplied in *E. coli*, extracted by Qiagen mini plasmid extraction kit, and sequenced at the Core Facility, University of Utah. The protocol for the recombinant plasmid synthesis is furnished in the Supporting Information.

Cell Culture. HeLa cells were cultured in Dubelco's DMEM cell culture medium with 10% FBS and maintained under 5% CO_2 in an incubator. Corning 75 and 225 mL cell culture flasks were used for growing cells. Water in the incubator pan was always autoclaved before use. The cells that were used in the experiments were between passage numbers 5 and 12. Cell confluence was kept between 50% and 70% for all RNAi assays.

RNAi Assay. During the day of the experiment, cells within the proper confluence level (~60%) were trypsinized and detached from the flask. Excess medium was added to inactivate trypsin. Cells were then centrifuged and resuspended in fresh medium. Cells were counted manually by using a standard hemocytometer and diluted so that 6000 cells are present per 80 μL of medium. Cells were kept in the incubator at 37 °C for about 45 min, while siRNA transfection

complexes were being formed. siPORT NeoFX transfection agent was used as the siRNA delivery method in all cell culture studies. All siRNAs and plasmids were diluted in Opti-MEM reduced serum medium, and siRNAs and plasmids were mixed together; similarly, transfection agents were diluted in Opti-MEM, and transfection complexes were allowed to form. Next, siRNA−plasmid mixtures are added into diluted transfection agent solutions and mixed thoroughly by pipetting. In all of the experiments, 96-well plates were used, and 20 μL of siRNA−transfection complex was discharged into each well. The cell suspension was then taken out of the incubator, and 80 μL of the cell suspension (containing 6000 cells) was added to each well. The plate was shaken several times and tilted several times to ensure homogenization of the two solutions. Next, the plate was kept inside the incubator, and within 4 h, the transfection of the cells was complete. Cells were allowed to grow for 36 h and were assayed for caspase 2 mRNA insert silencing using the Dual-Glo Luciferase Assay System from Promega. Initially, firefly luciferase emission was recorded, and then by adding the Dual Glo Stop and Glo reagent, the firefly luciferase was terminated and Renilla luminescence was recorded. Ratios of Renilla to firefly luminescence of samples were used to compute percent expression of caspase 2 insert mRNA. Each data point is the average of six independent experiments, and error bars represent standard deviation from the average value.

SiRNA−PKR Binding Assay. U87 cells (approximately 8×10^6 in a 75 cm^2 flask) were treated 24 h before lysis with human interferon-α A (PBL Interferon Source) to a final concentration of 1×10^6 U L^{-1}. The interferon-treated cells were washed twice with PBS and lysed by shaking with 3 mL of solubilization buffer (50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100), supplemented with protease inhibitor cocktail (ProteoBlock, Fermentas) for 30 min on ice. The lysates were clarified by centrifugation at 14 000g at 4 °C for 20 min and used directly in pull-down experiments. Magnetic streptavidin beads (0.5 mg, 50 μL) (Dynabeads M-280, Invitrogen) were prepared for RNA manipulation according to the manufacturer's protocol. Briefly, beads were suspended in 350 μL of binding buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl) and coated with 100 pmol of siRNA (100 μL) by gently rocking for 30 min at room temperature. The siRNA-coated beads were washed twice with binding buffer (500 μL) and once with solubilization buffer (500 μL) and incubated with 0.5 mL of cell lysate for 20 min at room temperature. Beads without siRNA were also incubated with cell lysate and served as a control. After incubation, the beads were washed four times with 500 μL of wash buffer (20 mM HEPES, pH 7.9, 2.5 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 0.5 mL DTT, 0.2 mg/mL yeast RNA, 0.2 mg/mL salmon sperm DNA). Bound protein was eluted from the beads by heating in loading buffer, separated on 6% SDS-PAGE, and transferred to a PVDF membrane for Western blotting. Membranes were blocked with blotting-grade milk (Bio-Rad), incubated with PKR antibody (Santa Cruz Biotechnology, 1:1000 dilution), washed with TBS-Tween, and incubated with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotechnology, 1:2000 dilution). The proteins were detected using ECF substrate (GE Healthcare) on a Typhoon Trio Variable Mode Imager (GE Healthcare), and band intensities were quantified using ImageQuant software (Molecular Dynamics). PKR binding affinity is reported as the average ratio of band intensities of modified siRNA to native siRNA for three independent experiments.

■ ASSOCIATED CONTENT

Supporting Information

Complete details of syntheses of modified phosphoramidites, proton and carbon NMR spectra, figure of the caspase 2 inserted sequence, and ESI−MS masses of the modified guide strands. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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