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8-Oxoguanosine Switches Modulate the Activity of Alkylated siRNAs by Controlling Steric Effects in the Major versus Minor Grooves

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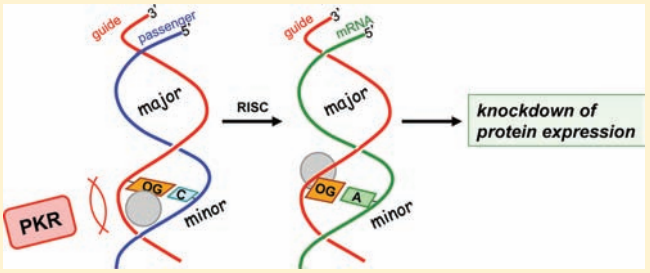
Supporting Information

ABSTRACT: Small interfering double-stranded RNAs have been synthesized bearing one or more base modifications at nucleotide positions 4, 11, and/or 16 in the guide strand. The chemically modified base is an N^2-alkyl-8-oxo-7,8-dihydroguanine (alkyl = propyl, benzyl) that can alternatively pair in a Watson−Crick sense opposite cytosine (C) or as a Hoogsteen pair opposite adenine (A). Cellular delivery with C opposite led to effective targeting of A-containing but not C-containing mRNA sequences in a dual luciferase assay with RNA interference levels that were generally as good as or better than unmodified sequences. The higher activity is ascribed to an inhibitory effect of the alkyl group projecting into the minor groove of double-stranded RNA preventing off-target binding to proteins such as PKR (RNA-activated protein kinase).



INTRODUCTION

Realization of the therapeutic potential of the RNA interference (RNAi) pathway[1−3] for reduction in expression of specific proteins has been hampered by problems associated with both intracellular delivery as well as off-target protein binding.[4−6] Even if sufficient concentrations of small interfering RNA (siRNA) duplexes can be introduced into the cell, their efficacy may be diminished or even rendered harmful due to off-target effects. These undesirable events include misloading of the siRNA passenger strand into the RNA-induced silencing complex (RISC), partial complementarity of the siRNA with mRNA sequences derived from other genes, and stimulation of the innate immune system by binding to toll-like receptors or other immunostimulatory proteins.[7−9] At a minimum, the potency of an siRNA is reduced whenever it is diverted to double-stranded RNA-binding (dsRB) proteins other than the RISC.[10]

RNA-dependent protein kinase (PKR) is a component of the interferon signaling system and is one example of a protein that participates in sequence-independent binding to siRNAs.[11] When PKR is activated by double-stranded RNA, antiviral signaling events are initiated that may be contrary to the goals of the desired RNAi therapy. Thus, a mechanism that would prevent binding of siRNAs to PKR and other dsRB proteins, yet would still permit correct loading and mRNA cleavage in the RISC, would increase the effectiveness of therapeutic siRNAs.

Chemical modification is recognized as a major strategy for improving the properties of siRNAs.[12−22] Many changes are tolerated in the passenger (sense) strand of siRNA. Alterations to the sugars or backbone of RNA strands can improve stability, reduce nuclease digestion, and increase cellular uptake.[23−25] Base modifications, although less common, are also being explored as a means to fine-tune interstrand interactions and to modulate the properties of the major and minor grooves.[19,26−29]

Our laboratories have been interested in the ability of purines bearing sterically blocking groups placed in the minor groove to prevent binding by dsRB proteins such as PKR.[30−32] Many cellular dsRB proteins interact with dsRNA via minor groove contacts, and binding of these proteins to siRNAs can be controlled by altering the minor groove structure.[30,32] However, we have also shown that minor-groove modifications can inhibit activity of the guide strand in RISC depending on the nucleotide position of modification.[30] Because of this, it would be beneficial to develop a method for shifting steric groups away from the minor groove when the guide strand enters RISC. We postulate that such a switch could be constructed by incorporating modifications that shift a sterically demanding substituent from the minor groove into the major groove within RISC (Figure 1). 8-Oxopurines appeared to us as potential frameworks for construction of such a switch due do their ability to exist in either syn or anti conformations depending upon the base opposite. For example, 8-oxo-7,8-dihydroguanine (OG) is well-known in the DNA damage literature as a base that pairs nearly equally well as a Watson−Crick partner with cytosine (C) or as a Hoogsteen pair with adenine (A).[33−35] In switching between the anti and syn conformations, the 8-oxo and N^2-amino groups exchange places between the major and minor grooves (Figure 2). Thus, we chose N^2-alkyl-8-oxo-7,8-dihydroguanosine derivatives[36] as first

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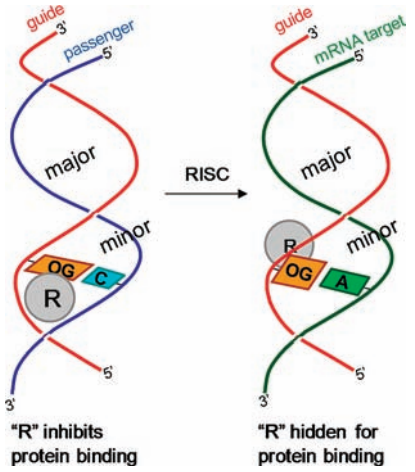

Figure 1. Design scheme for chemical modification of siRNA bases. An alkyl substituent "R" on the N^2 position of OG of the guide strand projects into the minor groove when paired opposite C, preventing binding to dsRB proteins such as PKR. When OG switches to Hoogsteen binding opposite A in the mRNA target strand in the RISC, the "R" group switches position to the major groove.

candidates for a study in which steric blockades in the form of an N^2-alkyl group might prevent binding to dsRB proteins like PKR when the OG is paired opposite C, but the alkyl group is switched to the deep major groove in the RISC when the OG in the guide strand encounters its target mRNA with an A opposite.

Recent work by one of our laboratories has shown that purine N^2 substituents oriented in the minor groove demonstrate position-dependent effects when incorporated into siRNAs. An N^2-benzyl group on guanines in the passenger strand retained RNAi activity while significantly reducing binding to PKR.[32] Similarly, introduction of N^2 substituents as large as a propargyl-clicked mannosamine or piperidine on a 2-aminopurine residue replacing adenosine in the passenger strand of siRNA indicated that these large substituents were well tolerated in positions 3 + 16 of the passenger strand and still effective in positions 9 + 14.[30] Placement at guide strand position 14 was effective, although position 2 was less well tolerated. Importantly, nearly all of these minor groove modifications lowered dsRB protein (i.e., PKR and ADAR1) binding levels as compared to those of unmodified siRNAs.[30] Thus, steric bulk in the minor groove can be effective at reducing dsRB protein binding; however, judicious placement of the modification is required to retain siRNA activity.

Crystal structures of the argonaute (Ago) protein responsible for mRNA cleavage in the RISC indicate that binding to the guide strand of siRNAs is principally through contacts to the sugar–phosphate backbone and not to the bases, accounting for its sequence-independent ability to bind dsRNA.[37] Despite the fact that sugar and phosphate backbone modifications are usually more detrimental when placed in the guide strand as compared to the passenger strand of siRNAs, chemical changes in the base structures can be tolerated in the guide strand as long as they are not overly helix-destabilizing. For example, studies of 5-propynyluridine in the guide strand in which the alkynyl group projects into the major groove indicate sensitivity to the steric bulk in the 5′ seed region, but a lesser effect was observed for base substitutions toward the 3′ end.[28] On the other hand, the smaller 5-methyluridine substitution led to increased activity when it replaced U in the guide strand.[28] Thus, subtle influences of substituents in the major and minor grooves of siRNAs can influence RNAi activity depending on the size and location of the alkyl group. In the present work, we report the activity of siRNAs that are delivered into the cell with one or more alkyl groups projecting into the minor groove to block dsRB protein binding, followed by switching of the alkyl groups into the major groove upon target mRNA binding in the RISC.

RESULTS

Design of siRNAs. We initially chose a portion of the human caspase 2 sequence shown in Figure 3 (native UA sequence) as a convenient target for study because the knockdown of this protein is nontoxic to HeLa cells. In the work reported here, the caspase 2 sequence was inserted into the 3′ untranslated region of the Renilla luciferase gene of a dual *Renilla*/firefly luciferase reporter plasmid that was cotransfected into HeLa cells.[30] RNAi-mediated knockdown of the *Renilla* luciferase activity could then be compared to the firefly luciferase activity as an internal standard.

We chose to investigate the effects of modification at three key sites of the guide strand, each of which is a U in the native sequence. U's were chosen for replacement with modified OGs because the mRNA target strand contains an A at this site; Hoogsteen base pairing of the modified OG with A would place the N^2 alkyl group in the major groove of the duplex as desired for switching off the steric blockade (see Figure 2). Position 4 of the guide strand lies in a critical part of the seed region that is important for mRNA target recognition. For example, a single-base bulge between positions 4 and 5 completely inhibits mRNA

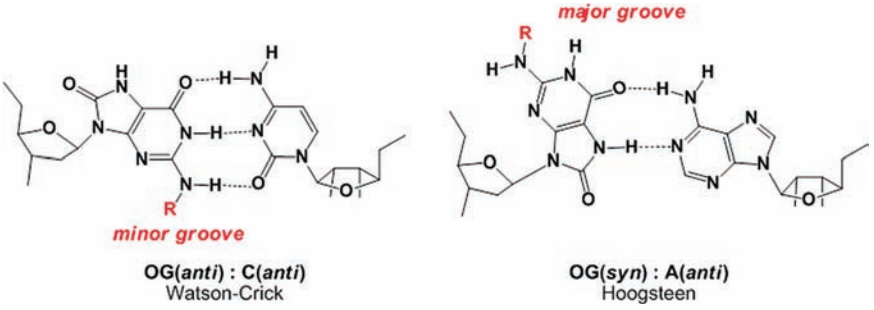

Figure 2. Base pairing scheme for 8-oxo-7,8-dihydroguanosine (OG) bearing N^2 substituents "R". Watson–Crick pairing of OG with C projects an N^2-alkyl group into the minor groove of double-stranded RNA (left). Opposite A (right), OG flips to the syn conformation for Hoogsteen pairing, projecting the R group into the deep major groove.

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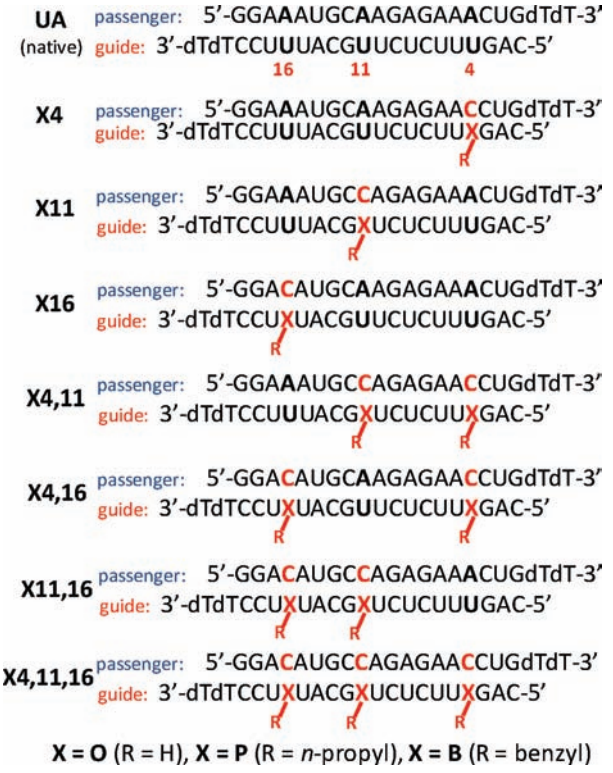

Figure 3. Sequences of modified siRNAs used in this work. U:A base pairs in the native caspase-2 sequence were replaced with X:C base pairs in which X is 8-oxo-7,8-dihydro-2′-deoxyguanosine bearing an N^2 substituent, and R = H (**O**), $CH_2CH_2CH_3$ (**P**), or $CH_2C_6H_5$ (**B**) at positions 4, 11, and/or 16 as shown.

cleavage by bacterial Ago.[38] Position 11 of the guide strand is another key position because the cleavage site is the phosphodiester bond opposite nucleotides 10−11. Interestingly, Ago tolerates the insertion of a single-nucleotide bulge at this site, but base mismatches diminish the cleavage activity.[38] Position 16 is in the 3′ portion of the guide strand that is anchored in the PAZ domain of Ago; insertion of base mismatches or bulges in this region have little effect on target cleavage.[38]

Accordingly, 21-mer siRNAs with two-nucleotide 3′ overhangs were synthesized using the corresponding modified phosphoramidites.[36] Previous thermal denaturation studies in which the OG modifications were paired opposite either C or A indicated modest decreases in thermal stability for each modification introduced.[36] T_m values for singly modified duplexes were approximately 60 °C; doubly and triply modified strands had T_m values near 55 °C with a shallow trend correlating the size of the R substituent on OG with decreasing stability: R = H (**O**) > R = propyl (**P**) > R = benzyl (**B**). All of the duplexes appeared sufficiently stable to warrant further investigation for RNAi activity. Alkylated OG bases were incorporated into RNA strands as 2′-deoxynucleosides because the absence of the 2′ hydroxyl is thought to facilitate the syn−anti interconversion.[39]

RNAi Knockdown Studies with Single Modifications in the Guide Strand. The siRNA duplexes shown in Figure 3 were evaluated for knockdown of *Renilla* luciferase reporter expression and normalized to constitutively expressed firefly luciferase using a dual reporter plasmid that was cotransfected into HeLa cells. The results are shown in Figure 4 for siRNA duplexes containing a single modification at position 4, 11, or 16 of the guide strand. Three OG modifications were evaluated at each position in which the N^2-alkyl substituent R was either H (strands labeled **O**), *n*-propyl (strands labeled **P**), or benzyl (strands labeled **B**), and then compared to protein expression using the native caspase 2 siRNA sequence (labeled **UA**). Each study was carried out in triplicate at three different concentrations: 100 nM, 1 nM, and 10 pM.

Duplexes containing an unalkylated OG (Figure 4A, **O4**, **O11**, and **O16**) were nearly as effective as **UA** at high concentration (100 nM), more effective at intermediate concentration (1 nM), and about the same as **UA** at very low concentration (10 pM). These data suggest that the presence of a single OG:A Hoogsteen pair in the RISC does not diminish the activity of hAgo2, the enzyme responsible for mRNA cleavage in HeLa cells, and indeed may even enhance either RISC loading or Ago-mediated cleavage. In addition, a single 2′-deoxyribonucleotide in the guide strand is not detrimental to knockdown activity, consistent with previous studies.[19]

Addition of a propyl group to N^2 of OG had position-dependent effects as shown in Figure 4B. When the modification was present at positions 11 or 16, the siRNA was twice as effective

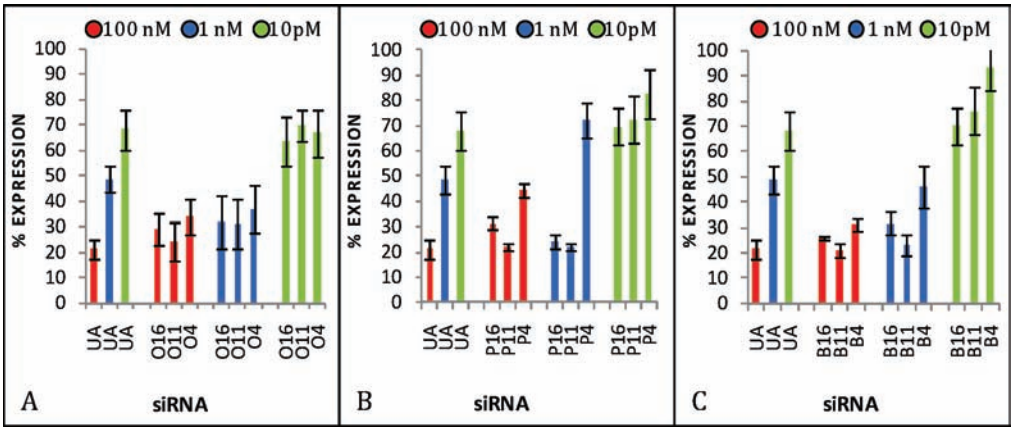

Figure 4. Expression of *Renilla* luciferase normalized to firefly luciferase for singly modified siRNAs. Sequences of siRNA duplexes are shown in Figure 3 in which **UA** is the native caspase 2 sequence, **O** has an 8-oxo-7,8-dihydro-2′-deoxyguanosine:C base pair replacing the U:A pair at positions 4, 11, or 16 of the guide strand, and **P** and **B** represent N^2-propyl and N^2-benzyl substituents on **O**. Experiments were conducted at 3 concentrations: 100 nM (red), 1 nM (blue), and 10 pM (green).

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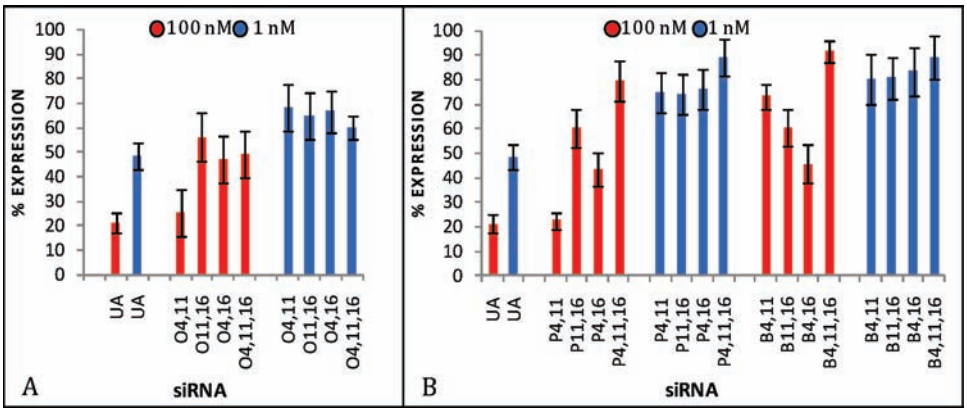

Figure 5. Expression of *Renilla* luciferase normalized to firefly luciferase for doubly and triply modified siRNAs. Sequences of siRNA duplexes are shown in Figure 3 in which **UA** is the native caspase 2 sequence, **O** has 8-oxo-7,8-dihydro-2′-deoxyguanosine:C base pairs replacing the U:A pairs at positions 4, 11, and/or 16 of the guide strand, and **P** and **B** represent N^2-propyl and N^2-benzyl substituents on **O**. Experiments were conducted at two concentrations: 100 nM (red) and 1 nM (blue).

as the native sequence **UA** at reducing protein expression at 1 nM concentration (Figure 4B, **P11** and **P16**, blue bars). On the other hand, introduction of N^2-propyl-OG in position 4 of the guide strand significantly disrupts the seed region, resulting in higher reporter expression (Figure 4B, **P4**, red and blue bars).

The positional effects were similar for the benzyl substituent; steric bulk added to position 4 was less effective at knockdown than at positions 11 and 16 (Figure 4C). Overall, however, the worst N^2-benzyl modification (**B4**) was still nearly as effective as native **UA**, and the **B11** and **B16** duplexes showed higher levels of knockdown as compared to **UA** in the intermediate concentration range (1 nM). These data suggest that addition of a single alkyl group, either propyl or benzyl, to a modified purine residue can enhance the efficacy of an siRNA. This enhancement could be due to a higher effective concentration delivered to RISC if the alkyl group is acting as designed to block minor groove contacts to-off target RNA binding proteins.

RNAi Knockdown Studies with Multiple Modifications in the Guide Strand. Next, we investigated the effects of introducing two or three N^2-alkyl-OG modifications on the ability of siRNAs to inhibit protein expression. As indicated in Figure 3, U:A base pairs of the native sequence were replaced with X:C base pairs to yield variants at positions 4 + 11, 4 + 16, 11 + 16, and 4 + 11 + 16. Luciferase expression levels obtained from these experiments are shown in Figure 5. In general, substitution of a U in two or three positions of the guide strand with an OG (Figure 5A) was detrimental to knockdown activity with one exception: the **O4,11** siRNA was nearly as effective as the native **UA** duplex. Interestingly, the same trend was observed with the propyl modifications. **P4,11** retained native-like activity at the higher concentration level (100 nM), although knockdown activity was diminished at 1 nM (Figure 5B). On the other hand, multiple substitutions with the benzyl substituent compromised the activity of siRNA more severely (Figure 5B).

Requirement for Switching to the Major Groove for RISC Activity. Next, we examined whether it was necessary to switch the alkyl group into the major groove. That is, can the N^2-alkylated siRNAs still lead to effective knockdown if the projection of Pr or Bz remains in the minor groove when the guide strand binds to the mRNA strand? To examine this, we constructed a mutant target RNA sequence with C instead of A opposite position 11 of the guide strand. The efficiencies of knockdown of protein expression by the original unmodified sequence (UA) as well as all singly modified siRNAs at position 11 against both caspase-2-containing target (A opposite position 11) and the mutant target (C opposite position 11) at concentrations of 100 and 1 nM are shown in Figure 6. In this set of studies, all siRNAs were delivered as Watson−Crick based paired duplexes. First, we note that the efficiency of RNA interference by an siRNA containing a guide-strand U at position 11 is reduced as compared to the native sequence, as expected for the formation of a single-base mismatch, U:C, in the RISC. Second, the effect of unalkylated 8-oxoguanosine remaining as a Watson−Crick base pair at position 11 (Figure 6, O11:C) is to modestly decrease the knockdown efficiency. This is somewhat surprising, because the thermal stability of O11:C is about 2 °C higher than that of O11:A.[36] Whether this is due to the greater base stacking of the OG(syn):A(anti) base pair or a destabilizing interaction created by the C8 oxo group of the OG(anti):C(anti) base pair is not known. Importantly, both the propyl and the benzyl substituents added to the O11 nucleotide of the guide strand led to very significant decreases in knockdown efficiency when they remained in the minor groove due to Watson−Crick pairing to the opposite C nucleotide of the mRNA target strand. For example, at the 1 nM concentration level, the percent of protein expression increased 3-fold for P11:C and B11:C as compared to P11:A and B11:A (Figure 6).

PKR Binding Studies. The protein expression studies described above indicated that certain single or double guide strand modifications in a caspase 2 siRNA sequence could lead to knockdown that was equal to or greater than that of the native strand. To test the switching mechanism that projects sterically encumbering groups in either the minor or the major grooves, we examined the ability of the selected duplexes to bind to the dsRB protein PKR. For these studies, we generated 5′ biotinylated passenger strands and used affinity purification with magnetic streptavidin beads to isolate dsRB proteins in U87 cell lysates as previously described.[30] The isolated protein was then quantified by Western blotting, allowing us to determine the effect of the modifications on PKR binding.

We expected that binding would be highly dependent upon the identity of the base opposite the alkylated 8-oxoguanine nucleotide; cytosine opposite should inhibit PKR binding due to disruption of minor groove contacts, in comparison to A opposite,

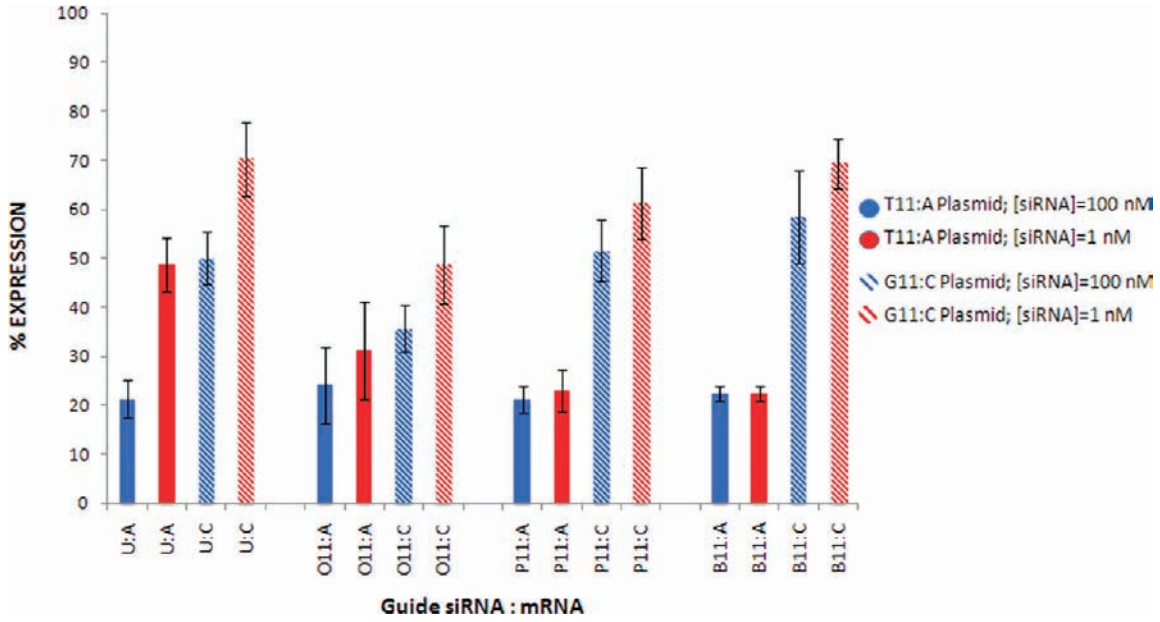

Figure 6. Expression of *Renilla* luciferase normalized to that of firefly luciferase for singly modified siRNAs at position 11 targeted to A versus C in the mRNA sequence. Sequences of siRNA duplexes are shown in Figure 3 in which **U:A** is the native caspase 2 sequence, **O** has 8-oxo-7,8-dihydro-2′-deoxyguanosine:C base pairs replacing the U:A pairs at position 11 of the guide strand, and **P** and **B** represent N^2-propyl and N^2-benzyl substituents on **O**. Experiments were conducted at two concentrations: 100 nM (red) and 1 nM (blue). The *x* axis depicts the anticipated base pair at position 11 when the guide strand is bound to mRNA of native (solid) or mutant (hashed) sequences.

which is the mRNA base targeted in the RISC. Indeed, this trend was observed for both the **P4** and the **B4** duplexes (Figure 7A), despite the fact that the 4 position was less effective at knockdown than were single guide-strand modifications at 11 or 16. The N^2-propyl group was detrimental to PKR binding whether 8-oxoguanosine was in either the anti or the syn conformation (C vs A opposite); however, PKR binding was minimal (20%) when the base analogue was in a Watson−Crick base pair (opposite C, propyl group was in the minor groove) (Figure 7A, lane 3). The benzyl group did not appear to influence PKR binding as much as the propyl substituent, although the same trend was apparent (Figure 7A, lanes 4 and 5).

For the doubly modified siRNA **P4,11**, which was the most active siRNA of the multiply modified duplexes, the trend was even more dramatic. Propyl groups extending into the minor groove (P4,11:C) due to Watson−Crick pairing led to a 70% reduction in PKR binding (Figure 7B, lane 8), while switching the alkyl groups to the major groove (**P4,11:A**) via Hoogsteen pairing actually enhanced PKR binding by almost 30% as compared to unmodified RNA (Figure 7B, lane 7). Overall, there was a 5-fold difference in PKR binding to **P4,11** depending on whether the propyl groups were in the major or minor grooves.

DISCUSSION

Double-stranded RNA binding proteins such as PKR are present at significant intracellular levels and thus compete with the RISC loading complex (RLC) for capture of siRNAs that enter the cell.[11,32] We designed an 8-oxoguanosine-based switch to turn on and off steric blockades to dsRB protein binding in the form of N^2-alkyl groups on the purine base. OG is well studied in duplex DNA, and its dual conformational behavior, anti when paired with C and syn when paired with A, is supported by T_m, NMR, crystallographic, and polymerase insertion studies.[34,35,40,41] A more limited number of studies of OG in dsRNA or in A-form DNA:RNA helices suggests the same conformational behavior.[39,42]

Our previous work described the incorporation of N^2-alkyl groups on 2-aminopurine bases replacing selected adenosines in both the passenger strand and the guide strand; no anti−syn switching was anticipated for these modifications. Modifications to the passenger strand were well tolerated; however, guide strand position 2 was quite sensitive to the addition of minor groove modifications while steric bulk added to guide strand position 14 led to siRNAs that were nearly as active as the native sequence.[30] siRNAs containing two sites of minor groove modification in the passenger strand were particularly effective at disrupting dsRB protein binding.[30,32] Therefore, a key goal of the present work was to amplify the utility of base modifications in the minor groove to block dsRB protein binding by designing a mechanism to switch off the steric effects when the guide strand was bound to the RISC. To do this, siRNA duplexes were prepared containing alkylated 8-oxoguanosines, Watson−Crick paired for delivery into the cell, but targeted to adenosines sites in mRNA such that Hoogsteen base pairs would be present in the RISC. In the latter arrangement, we proposed that the bulky alkyl group would be accommodated in the deep major groove of dsRNA.

As a general observation, we found that modifications to a single site of the guide strand led to RNA interference activity that was as good or better than the native caspase 2 sequence. The presence of an OG(syn):A(anti) base pair had little effect on the ability of the dsRNA to enter the RISC and target the appropriate mRNA site as demonstrated by the data shown in Figure 4A in which unalkylated **OG**-containing strands behave similarly to the native sequence. These results suggest that all three regions of siRNAs, the seed region, the cleavage site, and the 3′ end of the guide strand, can tolerate unusual modes of base

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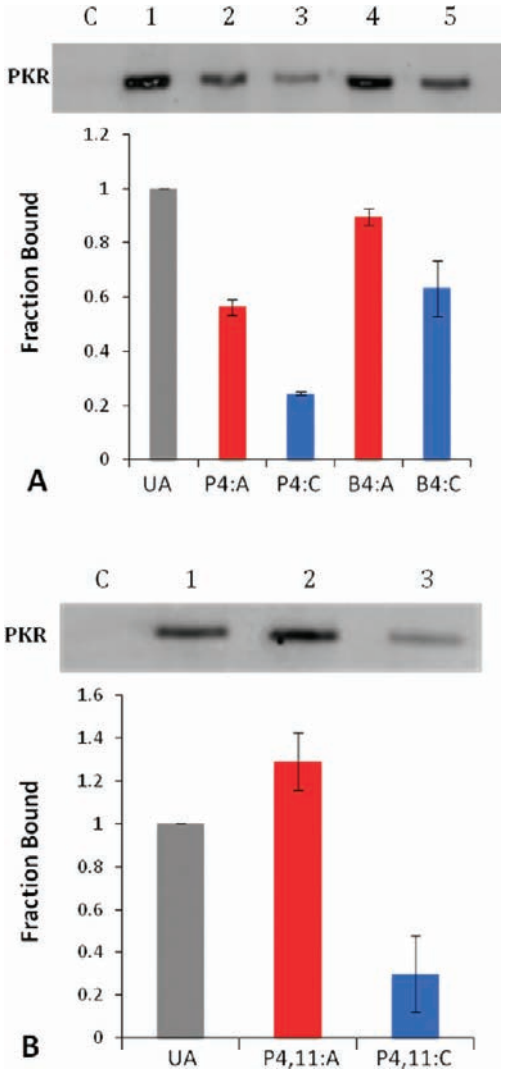

Figure 7. PKR binding assay with chemically modified siRNAs containing 8-oxoguanosine switches. Guide strand sequences are shown in Figure 3 with the N^2-alkyl groups propyl (P) or benzyl (B) extended in the minor groove when paired opposed C to inhibit PKR binding (blue bars); switching the alkyl group to the major groove (opposite A, red bars) leads to increased PKR binding. (A) siRNA duplexes singly modified at position 4 of the guide strand, along with a representative Western blot. No siRNA control. (1) UA (unmodified RNA). (2) P4:A. (3) P4:C. (4) B4:A. (5) B4:C. (B) siRNA duplexes doubly modified at positions 4 and 11 of the guide strand C. No siRNA control. (1) UA. (2) P4,11:A. (3) P4,11:C.

pairing within the RISC. When alkyl groups are added to form the **P** and **B** series of modifications, the protein expression levels remain low, particularly at the 1 nM concentration (Figure 4B,C), with the exception of **P4**, which was the least effective location for modification.

To verify that the steric blockade in the minor groove needed to be switched off at the stage of the RISC by flipping the propyl or benzyl group to the major groove, we tested the position 11 modified siRNAs with a mutant plasmid containing C opposite this site. For both the N^2-propyl and the benzyl substituents, allowing the group to remain in the minor groove was detrimental to protein knockdown. Taken together, the results with the two target sequences, native versus C mutant, validate the hypothesis that switching the steric blockade into the deep major groove during mRNA binding is beneficial to knockdown efficiency. Overall, these data suggest that the switching mechanism may be working as intended; the effective concentration of the siRNA should be higher if the alkyl groups are preventing the off-target diversion of dsRNA to PKR or other factors, thus leading to lower levels of protein expression. Consistent with this, the PKR binding assay indicated that alkylation of position 4 with either an N^2-propyl or an N^2-benzyl group reduced PKR binding, particularly when the alkyl group was projected in the minor groove via Watson−Crick pairing opposite C (Figure 7A). It is interesting to note that the smaller propyl group had a larger effect on inhibition of PKR binding than did the benzyl group (Figure 7A). It is not clear why this was the case, although it may suggest an incomplete switching mechanism. Regardless, additional experiments will be necessary to clarify this matter, and additional substituents should be studied to fully understand the effects of perturbations of the major and minor grooves of dsRNA during dsRB protein binding.

A surprising result was found in the case of **P4,11**. While the **P4** modification was the least successful of the singly modified siRNAs at knockdown of protein expression, the addition of a modification to position 11 seemed to rescue the interference activity, making **P4,11** the most active of the multiply modified siRNAs (Figure 5B). This cannot be due to high activity of the **P11** site alone, because **P11,16** is not particularly active. This result suggests the possibility of synergistic features of these multiple modifications that are not yet fully understood. Indeed, the PKR binding data for **P4,11** are particularly dramatic, supporting the switching mode between low PKR binding when the propyl groups are projected in the minor groove (cytosines opposite OGs) and 5-fold higher binding to PKR (Figure 7) when the propyl groups are in the major groove (adenines opposite OGs).

An additional factor to consider is whether thermal destabilization of regions of the siRNA duplex is beneficial or harmful to siRNA activity. Pertinent to this point, Addepalli et al. have shown enhancement of siRNA potency by incorporation of mismatches near the cleavage site, and they postulate that this effect may be due to increased efficiency of RISC loading.[43] In previous work, we showed that the incorporation of one, two, or three N^2-alkyl-OG bases had moderate, incremental effects on the thermal stability of 21-mer siRNA duplexes.[36] The lowering of the T_m value was most pronounced for modifications at position 4 of the guide strand (T_m's in the order 16 > 11 > 4), for the larger alkyl group (H > Pr > Bn), and for Hoogsteen pairing versus Watson−Crick pairing (C opposite > A opposite). Thermal stability may be one of many factors governing the RNAi activity of the singly modified siRNAs. For example, the O, P, and B bases opposite A showed consistently 3−4 °C additional lowering of the T_m when the modification was in position 4 as compared to position 16. This suggests a subtle influence of surrounding base context that might disrupt the helical pairing and stacking to a small extent. When this disruption occurs in the seed region of siRNA, it is expected that RNAi activity will be diminished, as was observed for **P4** and **B4**.

To gain further insight into the role of N^2-alkyl substituents on purines in selected locations in the RISC, we examined a crystal structure from the Patel laboratory in which a DNA:RNA duplex fills the dsRNA binding region of a bacterial Ago from *T. thermophilis* (PDB code 3HVR).[44] The enzyme was inactivated by a single mutation in the active site (D478N), and the structure included a 21-nt DNA guide strand hybridized to a 19-nt RNA

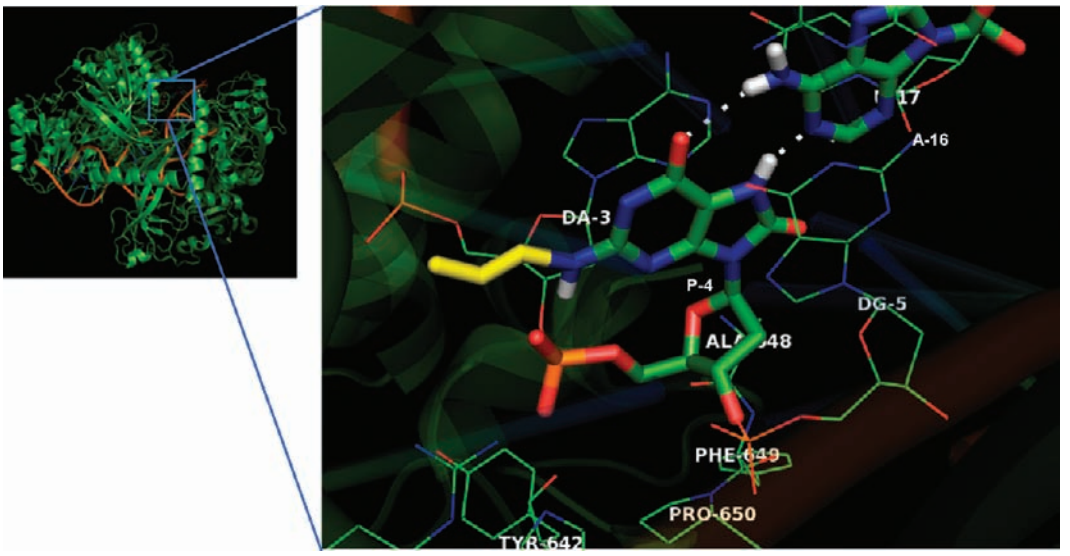

Figure 8. Modeling based on the crystal structure of *T. thermophilus* Ago D478N catalytic mutant in complex with 21-nt guide DNA and 19-nt target RNA, showing residues (labeled) within 5 Å of P-4 in the guide strand. The guide strand from the original crystal structure (PDB code 3HVR[44]) was modified at position 4 with P-4 (stick) in the syn conformation in place of G, and the target RNA was modified at position 16 with A (stick) in place of C. The N^2-propyl side chain is shown in yellow.

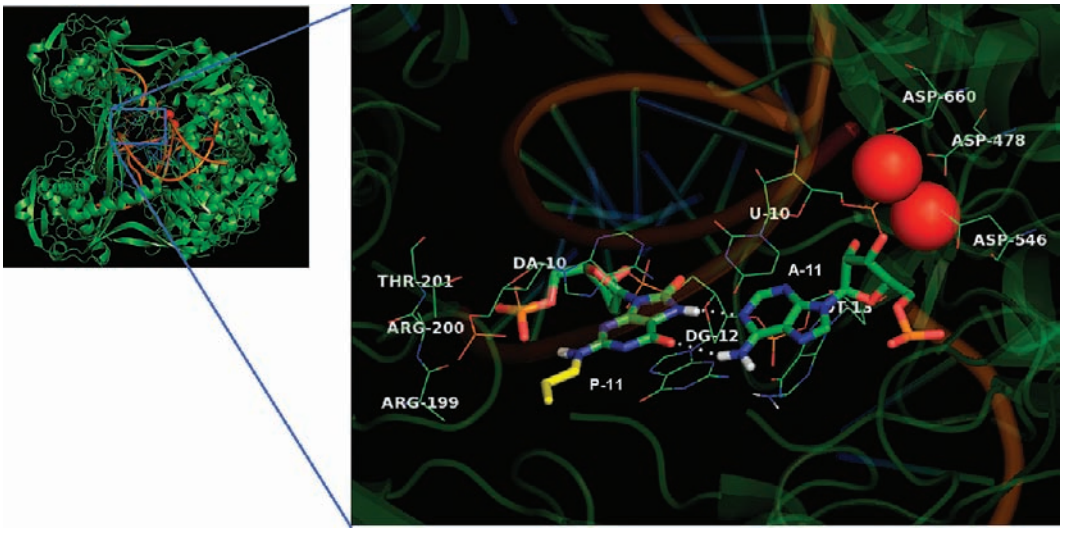

Figure 9. Modeling based on the crystal structure of *T. thermophilus* Ago D478N catalytic mutant in complex with 21-nt guide DNA and 19-nt target RNA, showing residues (labeled) within 5 Å of P-11 of the guide strand and also the cleavage site. The guide strand from the original crystal structure (PDB code 3HVR[44]) was modified at position 11 with P-11 (stick) in the syn conformation in place of G, and the target RNA was modified at position 11 with A (stick) in place of C. The N^2-propyl group (yellow) projects into a void. The two active site Mg^{2+} ions are shown as red spheres.

strand. We replaced the guanine bases at positions 4 (Figure 8) and 11 (Figure 9) of the guide strand with an N^2-propyl-8-oxo-7,8-dihydroguanine base and rotated it into the syn conformation. In addition, the passenger strand cytosines that were opposite were replaced with adenines to illustrate Hoogsteen base pairs at both sites. No additional computational modeling was performed, but the structures help to determine the space available for alkyl-OG(syn):A(anti) pairs.

Figure 8 shows a close-up of the P4:C base pair replacement (bold sticks), the amino acid and nucleotide residues within 5 Å of the modified base pair (lines), and the remaining portions of the backbone structure (ribbons). Although the overall sequence is different in the crystal structure as compared to our RNAi studies, it is clear from the figure that the modified base at position 4 (P-4 in Figure 8) can be accommodated in the syn conformation and remain stacked, in this case between deoxyadenosine (DA-3) and deoxyguanosine (DG-5), and oriented for hydrogen bonding to adenosine (A-16) in the target RNA strand. The propyl group (yellow) projects into the major groove and is apparently unrestricted. The closest amino acid residue to the propyl C1 carbon is a terminal nitrogen of Arg611 at a distance of about 5 Å. Another interesting feature is the relatively short distance between the N^2-hydrogen and an anionic phosphate oxygen (1.5 Å); this potential hydrogen-bond formation

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could either stabilize or distort the structure. If a hydrogen bond does form, it would help lock the 8-oxoguanosine in the syn conformation, as desired for correct pairing with A in the target mRNA. On the other hand, the seed region surrounding position 4 is typically extremely sensitive to duplex structure, and thus subtle effects that slightly distort the helix might play a role in reducing the activity of **P4** and **B4** siRNAs.

Figure 9 illustrates a similar replacement of N^2-propyl-8-oxo-7,8-dihydroguanine for the guanine base at position 11 (P-11 in Figure 9) along with the corresponding replacement of C with A-11 in the target strand. The N^2-propyl substituent projects into a large void in the major groove and appears completely unencumbered. The closest amino acid residue to the C1 carbon of the propyl group is the methyl group of Thr201 at a distance of >7 Å. The N^2-hydrogen is roughly 3 Å from a phosphate oxygen, and there are no close contacts with protein residues. In general, the region surrounding the P-11:A base pair is much less congested in the major groove than the area near P-4:A, suggesting that major groove modifications near the cleavage site may be more readily accommodated than near the seed region.

CONCLUSIONS

Chemical modification is a promising strategy to increase the potency and specificity of small interfering RNAs for therapeutic applications of the RNA interference mechanism. This work departs from most previous studies in focusing on guide strand rather than passenger strand modifications and in utilizing base rather than ribose or phosphate chemistry. Here, we add a new feature to the concept of base modification in siRNA through the design of a switchable base, 8-oxo-7,8-dihydroguanine, whose N^2-amino group can project into either the minor groove or the major groove depending on the base opposite, thereby controlling steric interactions with proteins at different stages of the RNAi process. These studies show that a Hoogsteen base pair formed by 8-oxoguanine in the syn conformation pairing with a target adenosine is tolerated in the seed region (position 4), at the cleavage site (position 11), as well as in the less critical 3′ region (position 16). The N^2-propyl and N^2-benzyl-8-oxoguanine bases project an alkyl group into the minor groove when Watson−Crick paired opposite cytosine. Using a dual luciferase reporter plasmid in HeLa cells and standard 1 nM concentrations of siRNA duplexes, we found that single site modifications of this type led to knockdown of protein expression that was more efficient than the unmodified siRNA in all cases except one, **P4**, which was similar to unmodified siRNA. Furthermore, we found that the switching of alkyl group to the major groove when the guide strand binds to the RISC is essential to obtain efficient knockdown of protein expression. A PKR binding assay supported the model that steric projections in the minor groove help prevent diversion of the modified siRNA to off-target protein binding. Switching of the alkyl group to the major groove of dsRNA led to greater protein binding and permitted Ago2 activity.

Although doubly and triply modified siRNAs were generally not very active for knockdown of protein expression, certain combinations, notably **P4**,**11**, displayed good knockdown properties as well as PKR binding characteristics that support the proposed switching mechanism for 8-oxoguanine. These first generation studies of minor versus major groove alterations to siRNA guide strands represent a promising lead for future optimization of their performance in RNA interference while preventing off-target protein binding.

EXPERIMENTAL METHODS

The N^2-alkyl-8-oxo-2′-deoxyguanosine phosphoramidites and oligoribonucleotides incorporating them were synthesized as previously described.[36]

siRNA Duplex Formation. Hybridization to form siRNA duplexes was accomplished by combining equal amounts of purified passenger and guide strands to a final concentration of 1 μM in 10 mM Tris-HCl, 100 mM NaCl, pH 7.5. The samples were heated at 95 °C for 5 min followed by cooling to room temperature over a period of approximately 8 h.

Cell Culture, Transfection, and RNAi Activity Assay. HeLa cells (ATCC) were grown at 37 °C in humidified 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were maintained in exponential growth. HeLa cells were reverse-transfected using siPORT NeoFX transfection reagent according to the manufacturer's instructions. Briefly, cells grown in flasks at approximately 60−80% confluence were detached with Trypsin-EDTA and diluted in fresh medium (DMEM, 10% FBS) to the required concentration for further use.

A vector was prepared from the psiCHECK2 plasmid (Promega) containing the reporter genes *Renilla* and firefly luciferase (hRluc and hluc+, respectively) with the caspase 2 siRNA sequence inserted into the 3′ untranslated region of the former (psiCHECK2- Cas2) (Figure S1). *Renilla* luciferase was used as a reporter and firefly luciferase was used as a control. An aliquot (0.5 μL) of siPORT NeoFx was diluted to 10 μL in Opti-Mem medium. An aliquot (80 μL) of the cell suspension (8000 cells/well) was added to 96-well plates. Cotransfections of the plasmid and siRNAs were performed with siPORT NeoFX in 96-well plates. Hybridization buffer was used as a negative control. Other negative controls including single-strand sense and antisense strands and a random duplex DNA sequence (18-mer) were also used. After 24 h, caspase-2 gene knockdown was analyzed using the Dual Luciferase Assay Reporter system (Promega) according to the manufacturer's instructions (Figure S2). Luciferase activity was measured with a multiplate luminometer, using an integration time of 2 s. RNAi activity was measured as the normalized ratio between hRluc and hluc+ and reported as the average of six independent experiments. The results were validated using a one-sample *t* test, and the full statistical details are provided in the Supporting Information (Tables S4 and S5).

PKR Binding Assay. U87 cells (approximately 8×10^6 in a 75 cm^2 flask) were treated 24 h prior 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 14 000*g* at 4 °C for 20 min and used directly in pull-down experiments. Magnetic steptavidin 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). The beads were then 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. Detailed sequence information of all oligomers, plasmid preparation, dual-luciferase assay protocol, T_m data, complete statistical analyses, and complete ref 25. This material is available free of charge via the Internet at http://pubs.acs.org.

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