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Structure-Based Design of Novel Chemical Modification of the 3′-Overhang for Optimization of Short Interfering RNA Performance

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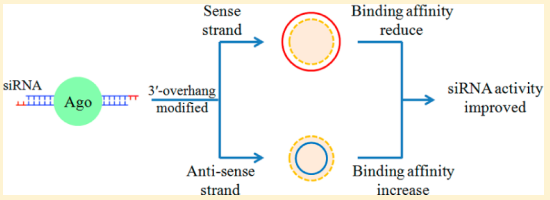
Supporting Information

ABSTRACT: Short interfering RNAs (siRNAs) are broadly used to manipulate gene expression in mammalian cells. Although chemical modification is useful for increasing the potency of siRNAs *in vivo*, rational optimization of siRNA performance through chemical modification is still a challenge. In this work, we designed and synthesized a set of siRNAs containing modified two-nucleotide 3′-overhangs with the aim of strengthening the interaction between the 3′-end of the siRNA strand and the PAZ domain of Ago2. Their efficiency of binding to the PAZ domain was calculated using a computer modeling program, followed by measurement of RNA−Ago2 interaction in a surface plasmon resonance biochemical assay. The results suggest that increasing the level of binding of the 3′-end of the guiding strand with the PAZ domain, and/or reducing the level of binding of the sense strand through modifying the two-nucleotide 3′-overhangs, affects preferential strand selection and improves siRNA activity, while we cannot exclude the possibility that the modifications at the 3′-end of the sense strand may also affect the recognition of the 5′-end of the guiding strand by the MID domain. Taken together, our work presents a strategy for optimizing siRNA performance through asymmetric chemical modification of 3′-overhangs and also helps to develop the computer modeling method for rational siRNA design.



Small interfering RNAs (siRNAs) are widely used to transcriptionally silence gene expression in mammalian cells. The typical siRNA is comprised of two complementary strands of 21 nucleotides (nt) with 2 nt overhangs at both 3′-ends.[1] Upon introduction into the target cell, one strand of the duplex RNA is recognized as the guiding strand (or antisense strand) and incorporated into the RNA-induced silencing complex (RISC), while the nonguiding strand (sense strand) is cleaved and released.[2,3] The RISC containing the guiding strand subsequently binds to and cleaves the complementary target mRNA, which results in sequence-specific inhibition of gene expression.[4]

Argonaute 2 (Ago2), the catalytic component of the RISC, plays a central role in this gene silencing process. Crystal structure analysis reveals that Ago2 has a conserved structure, including Piwi-Argonaute-Zwille (PAZ), MID, and PIWI domains.[5,6] The PAZ domain specifically recognizes siRNA through binding to the two-base overhang at the 3′-end of the strand.[7,8] The PIWI domain exhibits a secondary structure similar to that of RNase H and synergistically interacts with the 5′-end of siRNA to catalyze the separation of the two strands of siRNA.[9−11]

Although the use of siRNAs in therapeutics holds enormous potential, the application is hindered by several issues such as physical instability, side effects, and potency of siRNAs *in vivo*. Currently, chemical modification is generally considered a prerequisite for fulfilling the potential of siRNAs *in vivo*. The major objectives in the optimization of siRNA performance through chemical modification include improving siRNA potency and reducing its off-target effects. Selective incorporation of the guiding strand into the RISC is an essential step for improving RNA interference activity and avoiding off-target effects. It has been proposed that the relative stabilities of the base pairs at the 5′-ends of the two siRNA strands determine the strand selection.[12,13] However, recent studies showed that the terminal structures of siRNA are also involved in strand selection, i.e., the unilateral 2 nt 3′-overhang and the "fork−siRNA duplexes".[14,15] Furthermore, Masayuki et al. demonstrated that, because of preferential antisense strand selection, siRNAs with unilateral 2 nt 3′-overhangs on the guiding strand are more potent than siRNAs with symmetric overhangs.[16] This suggests that the terminal structure of siRNA is the primary factor in strand selection. Meanwhile, nuclear magnetic resonance (NMR) studies of the interaction of the PAZ domain with siRNAs showed that modifying the 3′-overhang, but not the double-stranded region, of the antisense strand improves the binding affinity.[17]

These results suggest that chemical modifications of the 3′-overhang, which increases the binding affinity of the 3′-terminus for the PAZ domain, have great impacts upon strand selection and thereby improve RNA interference activity and reduce off-target effects. In line with this hypothesis, several

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groups have measured the effect of aromatic modifications at the 3′-end of the guide strand on RNAi.[18,19] However, because of the lack of either detailed *in silico* analysis or experimental approaches for assessing the binding affinity of the 3′-terminus with the PAZ domain, the effect of chemical modifications of the 3′-overhang on siRNA activity remains undetermined.

In this work, we designed and synthesized a series of chemically modified nucleotide monomers containing an aromatic hydrophobic structure with the aim of strengthening the interaction between the 3′-end of the siRNA strand and the Ago2 PAZ domain. A set of modified 2 nt 3′-overhangs was assessed for its energy of binding to the PAZ domain using a computer modeling program. The results of *in silico* analysis were directly correlated to the binding affinity of the modified siRNAs with Ago2 *in vitro*. The results revealed that increasing the binding affinity of the 3′-end of the guiding strand with Ago2 and/or reducing the level of binding of the sense strand through modifying the 2 nt 3′-overhangs significantly improved RNA interference activity. Altogether, this work describes a method for optimizing siRNA performance through asymmetric chemical modification of 3′-overhangs and also puts forward a computer modeling method for rational siRNA design.

EXPERIMENTAL PROCEDURES

Cells and Plasmids. Hep G2 (Luc) is a strain genetically modified human Hep G2 cells stably expressing luciferase. MCF-7 cells, a human breast adenocarcinoma cell line, and human embryonic kidney 293 (HEK 293) cells were cultured in DMEM (Gibco), supplemented with 10% FBS and 1% penicillin/streptomycin. CMV-L1-neo^{RT} carries a copy of complete human LINE-1 DNA that bears a neomycin resistance gene as a reporter of LINE-1 retrotransposition.[20]

Calculation of the Energy of Binding of Modified siRNA Strands and Ago2. The crystal structure of the Ago protein PAZ domain [Protein Data Bank (PDB) entry 1SI2][21] was selected as the receptor model after extracting the ligand and adding the hydrogen of the protein in molecular modeling suite Schrödinger. The extracted original RNA strand ligand was added with hydrogen and kept as the template. The Embrace Minimization in MacroModel module (MacroModel, version 9.7, Schrödinger, LLC, New York, NY) was adopted to calculate the binding energy between the various ligands and the receptor and thus to evaluate and predict the relationship between the different modification of the siRNA 3′-end and the binding energy variation. The OPLS_2005 force field was applied, and the dielectric constant was set to 1.0 as the aqueous solution was considered. The PRCG method was applied, and the maximal number of iterations was set to 500. Other parameters were set to defaults.

Synthesis of Chemically Modified Nucleotide Monomers and siRNA. Compounds used to synthesize modified monomers were prepared as described previously.[22] The synthesis route is shown in the Supporting Information. The chemically modified monomers were introduced at the end of the strand through a coupling reaction, and the regular controlled pore glass (CPG) was adopted as the solid phase carrier (see the Supporting Information). All siRNAs targeting luciferase mRNA contain the same duplex region: 5′-UGAAGAGCCUGAUCAAAUA-3′ (sense strand) and 3′-ACUUCUCGGACUAGUUUAU-5′ (antisense strand). Each siRNA contains a chemical modification of 3′-overhangs as assigned (Table 2). The siRNAs targeting the *mdm2* gene contain the same duplex region: 5′-GCUUCGGAACAAGAGACCC-3′ (sense strand) and 3′-CGAAGCCUUGUUCUCUGGG-5′ (antisense strand). The siRNA targeting MOV10 gene contains the same duplex region: 5′-GAAACCCUGUGGUGACCAA-3′ and 3′-CUUUGGGACACCACUGGUU-5′ (antisense strand).[23]

Luciferase Reporter Gene Assay. Hep G2 (Luc) cells were seeded at a density of 9×10^3 cells/well in 96-well plates, and triplet assays were conducted at each dose. After incubation for 24 h, cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX reagent (Invitrogen). Cell lysates were harvested 48 h post-transfection. Luciferase assays were performed using the "Steady-Glo Luciferase Assay System" (Promega) according to the manufacturer's protocol on EnVision (PerkinElmer). Luciferase signals (sample) were normalized to cell number. All results were normalized to the control siRNA.

Sulforhodamine B (SRB) Assay. A sulforhodamine B (SRB) assay was optimized for the analysis of cytotoxicity and antiproliferative activity of chemically modified siRNAs in a 96-well format.[24] In brief, after incubation, cell monolayers were fixed with trichloroacetic acid (TCA) and stained. The protein-bound dye was dissolved in 10 mM Tris for the determination of the OD using a microplate reader. In the nonspecific toxicity assay, Hep G2 cells were seeded at a density of 9×10^3 cells/well in 96-well plates, and a triplet assay was conducted at each dose. After incubation for 24 h, cells were transfected with various amounts of siRNAs. The inhibition of cell growth was assessed 48 h post-transfection. To analyze the antiproliferative activity of chemically modified siRNAs, MCF-7 cells were used following a similar experimental procedure. The cytotoxicity for Hep G2 or inhibition of MCF-7 cell growth was calculated using the formula inhibition rates (%) = $[(OD_{492\ \text{control well}} - OD_{492\ \text{treatment well}})/OD_{492\ \text{control}}] \times 100\%$.

Surface Plasmon Resonance (SPR) Assay. Surface plasmon resonance experiments were performed on a BIAcore biosensor system (Biacore AB). In brief, the carboxymethylated surface of sensor chip CM5 was first activated with a mixture of *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) (Biacore AB). Subsequently, 35 μL of purified human Ago2 [>85% pure (Supporting Information)] at a concentration of 60 μg/mL was injected into flow cell 2 (FC2) for immobilization on the sensor surface. No protein was injected into FC1. Experiments were performed at 25 °C in HBS buffer [10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Nonidet P-40 (pH 7.4)] at a flow rate of 10 μL/min. To analyze the binding of siRNAs to Ago2, 30 μL of siRNAs (10 μM in HBS buffer) was injected, followed by passing the buffer over the chip and an elution step with 2 mM dithiothreitol in HBS buffer. Difference resonance spectra (FC2 − FC1) were recorded. Data were evaluated using BIAevaluation version 3.0 (Biacore AB). A plasmid expressing GST-tagged full-length human Ago2 protein was kindly provided by L. Joshua-Tor, and the protein was expressed in *Escherichia coli* and purified as described previously.[11]

Real-Time Polymerase Chain Reaction (PCR). MCF-7 cells or HEK 293 cells were transfected with siRNA (50 nM), and the total cellular RNA was extracted using Trizol reagent (Invitrogen) 48 h post-transfection. The amount of total RNA was determined using a UV spectrophotometer (BECKMAN DU800), and the data are used to normalize RNA template to generate cDNA. The mRNA levels were determined by

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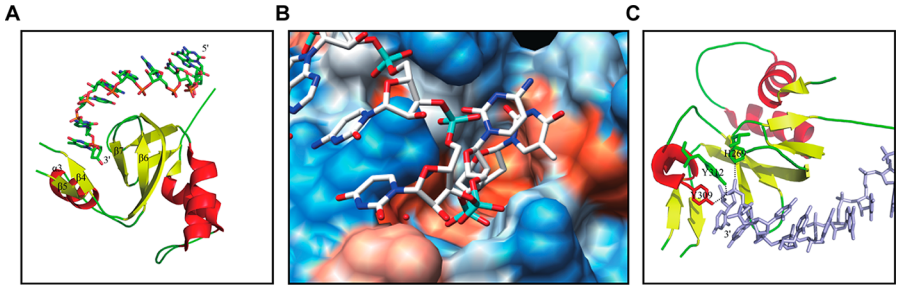

Figure 1. PAZ domain−siRNA-like duplex subcomplex. (A) View showing one PAZ domain that interacts with a siRNA-like end. (B) Electrostatic surface of the PAZ domain. The orange, blue, and white colors depict the hydrophobic, hydrophilic, and neutral regions, respectively. (C) Crystal structure of the PAZ domain of human Ago2.

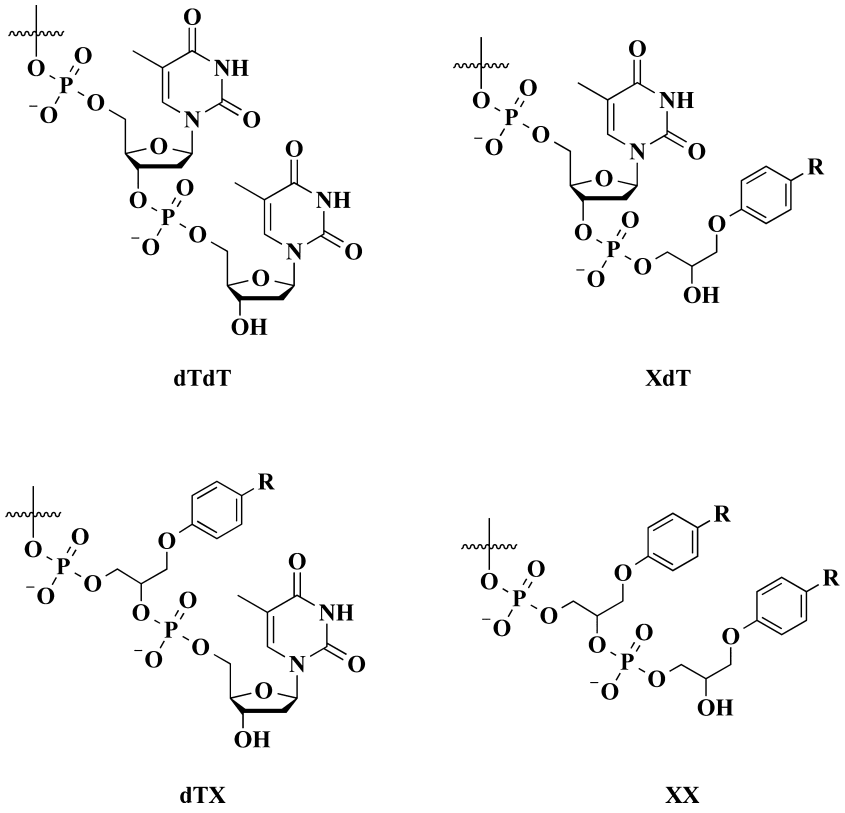

Figure 2. Structural overview of chemically modified nucleotide monomers and their combination. The designs of chemically modified X units are shown. For X_1, R = H. The structures of different chemically modified unit combinations are illustrated.

quantitative PCR (qPCR) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and the random hexamer-primed cDNA as a template according to the manufacturer's protocol. Results were expressed as the threshold cycle (Ct). The relative quantification of the target transcripts was determined by the comparative Ct method (ΔΔCt) according to the manufacturer's protocol. The GAPDH gene was used as reference to normalize data. The following primer sequences were used: MDM2, 5′-GAATCATCGGACTCAGGTACTC-3′ (forward) and 5′-TCTGTCTCACTAATTGCTCTCCT-3′ (reverse); MOV10, 5′-CGTCACCAAGACTCGGGTC-3′ (forward) and 5′-TCTTTTGCGGTTCAAATCCAGG-3′ (reverse); GAPDH, 5′-GGAGCGAGATCCCTCCAAAAT-3′ (forward) and 5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse).

Western Blot. MCF-7 cells or HEK 293 cells were transfected into siRNA (50 nM). Forty-eight hours post-transfection, cells were washed twice in cold PBS and extracted in 60 μL of RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 1% NP-40, 150 mM NaCl, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mM Na_3VO_4, and 1 mM NaF] on ice for 30 min. Protein samples were separated via 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore Corp., Bedford, MA). Membranes were blocked with 5% nonfat milk TBST buffer and then incubated with primary antibodies: monoclonal anti-MDM2 (Santa Cruz Biotechnology Inc.), anti-MOV10 (Cell Signaling Technology), or anti-β-actin (Sigma- Aldrich) for 1 h at room temperature. The membranes were washed and incubated for 1 h with HRP-conjugated anti-mouse or anti-rabbit secondary antibody (Cell Signaling Technology). Electrochemiluminescence was performed according to the manufacturer's instructions with the Fluor Chem TM HD2 system (ProteinSimple).

Cell Colony Assay. HEK 293 cells were seeded in six-well plates 1 day prior to transfection with 100 pmol of siRNA3.

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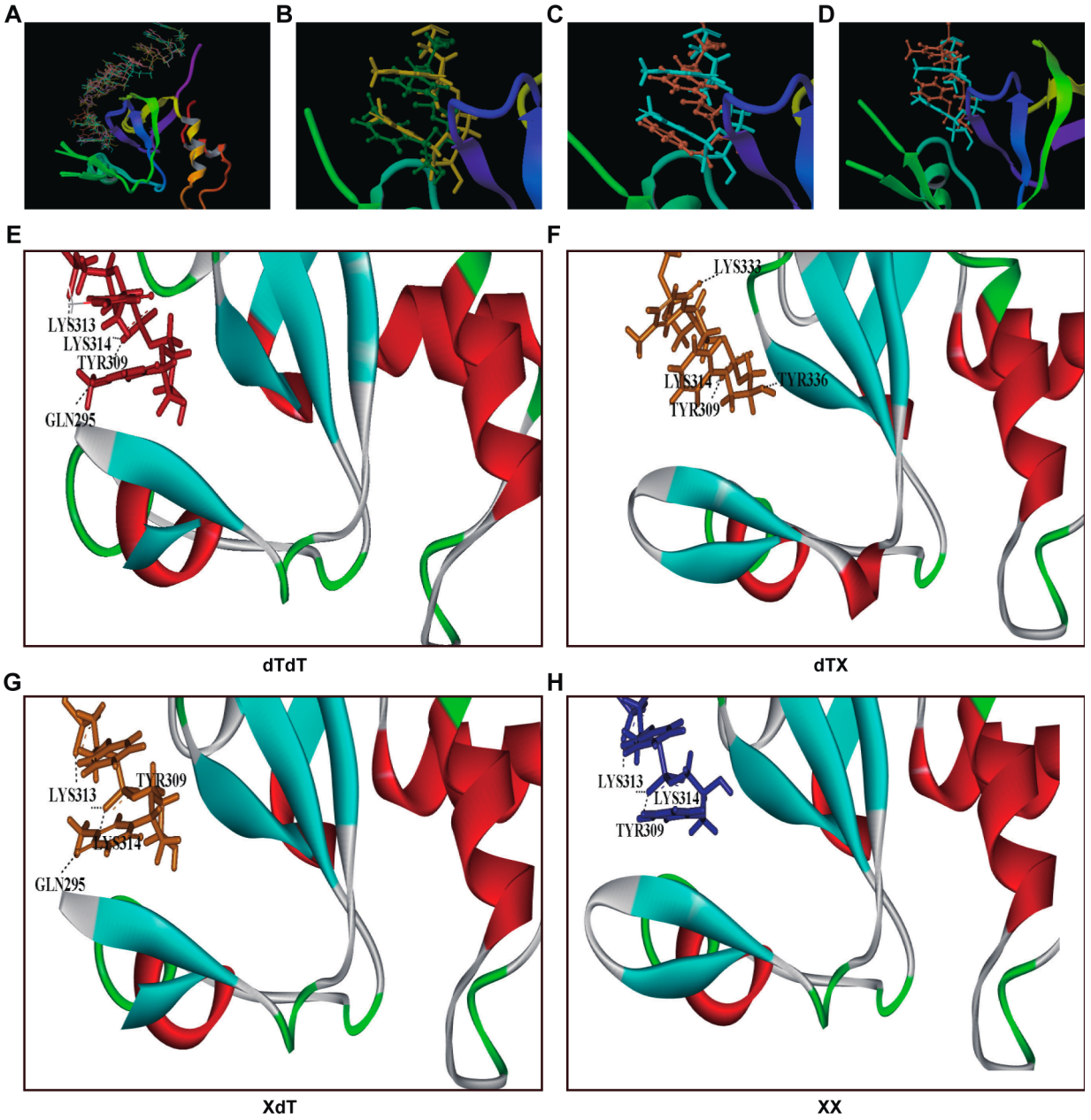

Figure 3. Interactions between the modified 3′-overhangs or the RNA strand and the PAZ domain. (A) Superposition of various siRNAs interacting with the PAZ domain. (B) Superposition of dTdT and dTX. dTdT and dTX are colored yellow and green, respectively. (C) Superposition of dTdT and XX. dTdT and XX are colored blue and brown, respectively. (D) Superposition of dTdT and XdT. dTdT and XdT are colored blue and brown, respectively. Panels E–H show the PAZ domain that interacts with the 3′-end of the RNA strand containing dTdT, dTX, XdT, and XX types of chemical modifications, respectively.

Twenty hours post-transfection, cells were transfected with 1 μg of Line-1 reporter DNA. Forty hours post-transfection, cells were detached from plates and seeded into six-well plates at serial dilutions. G418 (0.75 mg/mL) was then added to select for resistant cell colonies. Twelve days into selection when cell colonies were clearly visible, cell colonies were fixed with methanol at temperature for 10 min and stained with 0.5% crystal violet for 10 min.[20,25]

RESULTS

Interactions between the PAZ Domain of Ago2 and the 3′-End of siRNA. The molecular modeling suite Sybyl was adopted to analyze the structure of the Ago2 protein PAZ domain (PDB entry 1SI2), and the results are shown in Figure 1. The PAZ domain contains a β barrel and $\alpha\beta$ components that together form a conserved pocket structure (Figure 1A). The two nucleotides extending at the 3′-end of siRNA enter the pocket and interact with the residues of the PAZ domain through a hydrogen bond, p−π conjugation, or van der Waals interactions. Specifically, there are hydrophilic areas surrounding the groups linked to the phosphate backbone of siRNA, where residues His269, Tyr312, and Tyr309 form hydrogen bonds with the 3′-terminus of siRNA. The first nucleotide was almost buried in the pocket and was surrounded by the hydrophobic residues Leu294, Phe292, Leu337, Pro293, and Phe310. The second base was at the edge of the pocket with most the neutral or hydrophobic zone around it. The structural

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analysis suggests that it enhances the interaction between the bases at the dangling end and the residues at the PAZ domain upon introduction of the hydrophobic groups at the proper position of siRNA 3′-end bases.

Design and Synthesis of Chemically Modified Nucleotide Monomers and siRNAs. We next designed the chemically modified nucleotides that were used to synthesize siRNAs. The bioisosterism strategy was applied in our small molecular design, and the designed structures are shown in Figure 2. First, the pentose ring was opened and replaced by the straight chain retaining to simulate the strong hydrogen bond interaction between residues Tyr312 and Tyr309 and the original phosphate groups. Alternatively, the pyrimidine ring was substituted with the benzene ring to enhance the binding between the modified motif and the hydrophobic residues Phe292, Leu337, and Phe310 in the PAZ domain, thus strengthening the interaction between siRNA and the PAZ domain of Ago2 protein. Using the modified monomers described above, we designed a different modified combination at the 3′-terminus of siRNA. These combinations were classified into four types, namely, dTdT, XdT, dTX, and XX, which correspond to the first and second nucleotide beginning from the 3′-end of siRNA (Figure 2).

Binding Energy Calculation of the Chemical Modification Combinations. The four types of siRNA strands were first superimposed through the backbone to compare the conformational change of the overall structure and of the local modification sites of siRNA strands. The alignment results (Figure 3A) showed that the modification of the end nucleotides induces an only slight conformational change in the overall structure of the siRNAs, and all of the modified siRNAs specifically bind to the Ago2 protein in a similar configuration. However, as shown in Figure 3B−D, the conformations of the local modification sites of siRNA strands, upon binding to the PAZ domain active site, vary correspondingly with the change in the chemically modified combinations. The local conformation of the dTX or XX combination was consistent with that of the dTdT conformation to mimic the ribose five-ring structure with the linked phosphate groups, thus ensuring that the motif binds with the groove through forming hydrogen bonds. In addition, with regard to the dTX or XX combination, the pyrimidine structure was replaced with the hydrophobic group so that the benzene ring can enhance the hydrophobic interaction between the aromatic motif and the surrounding hydrophobic residues. The XdT modification resulted in a large structural distortion, which leads to a large difference in spatial conformation compared with that of the unmodified combination. Next, we assessed the binding energy between the 3′-end of siRNA and the PAZ domain (Figure 3E−H). The calculation results are listed in Table 1. The binding energies vary with different changes in the modified combinations at the 3′-end strand. The order of the mean total binding energies of various modified combination is as follows: dTdT > XdT > dTX ≈ XX (Table 1). This suggests that RNA strands containing XX or dTX at the 3′-end have a binding affinity for Ago2 protein higher than that of unmodified (dTdT) or XdT-modified RNA.

Measurements of the Binding Affinity of Chemically Modified siRNA and Ago2 Protein. We next synthesized the chemically modified monomer and siRNA according to our design. The synthesis procedure is given in the Supporting Information. The chemically modified monomers were further introduced at the end of the strand through coupling reactions, and the regular controlled pore glass (CPG) was adopted as the solid phase carrier.

Table 1. Predicted Binding Energies (ΔE) of the PAZ Domain with Different Chemically Modified Nucleotide Combinations (kJ/mol)

siRNA	valence energy	vdW	electrostatic	solvation	ΔE
dTdT	41.69	−81.65	−8068.27	7837.27	−270.95
dTX	60.24	−124.47	−8458.62	8165.93	−356.91
XdT	71.11	−48.62	−8479.88	8151.90	−305.49
XX	63.13	−106.49	−8562.41	8227.59	−378.18
dTX*[a]	72.73	−117.68	−8725.13	8410.89	−359.19
X*dT[a]	85.63	−41.49	−8512.94	8166.65	−302.15
X*X*[a]	64.16	−103.38	−8498.68	8184.11	−353.79

[a]X* represents the derivative that has a 4-fluoro substituent on the benzene ring.

After preparing the chemically modified siRNA, we first used surface plasmon resonance (SPR)-based biospecific interaction analysis to measure the binding affinity of siRNAs with purified Ago2 protein. The response unit (RU) values are shown in Figure 4A. The binding energy value (shown in Table 1) for each modification and RU value (shown in Figure 4A) of corresponding strand was normalized by maximizing the value to obtain the relative binding ability. Two sets of relative values for the chemical modification were plotted in Figure 4B. The data of *in silico* analysis were consistent with the binding affinity of the modified siRNAs for Ago2 that was measured by a surface plasmon resonance (SPR) assay *in vitro*. This result confirms the prediction based on *in silico* analysis.

Interfering Efficiency of Chemically Modified siRNA. We next evaluated the knockdown efficiency of siRNA containing the modifications described above in a luciferase reporter assay. siRNAs targeting the coding sequence of luciferase with and without the chemical modification were synthesized as described in Experimental Procedures are listed in Table 2. The Hep G2 cells stably expressing luciferase were transfected with each siRNA. The abilities of the siRNAs to silence luciferase gene expression were evaluated in a luciferase activity assay. All the siRNAs containing the chemically modified monomers exhibited potencies greater than that of the unmodified one (dTdT), suggesting the improved abilities of modified siRNA in gene silencing (Table 2).

When the modification of the 3′-overhang of the sense strand is of the XdT type, the dTX modification of the antisense strand always exhibits the greatest inhibitory effect regardless of the modified nucleotides used (Figure 5A). This suggests that the chemical modification, which strengthens the interaction between the 3′-end of the antisense strand and the PAZ domain, improves siRNA potency. Indeed, a similar result was realized when the dTX type of sense strands was used (Figure 5B), indicating that the improved siRNA potency resulting from the dTX type of antisense strand was independent of the modification of the sense strand. In contrast, the sense strand shows a higher binding affinity with Ago2-impaired RNA interference (Figure 5C). For example, the XdT type of sense strand showed an inhibitory effect stronger than that of the dTX type regardless the type of antisense strand or the modified nucleotide used. Taken together, the XdT/dTX (3′-overhang of the sense strand/antisense strand) modifications allow a higher binding affinity of the guiding strand and a lower binding affinity of the nonguiding strand for Ago2 and thus

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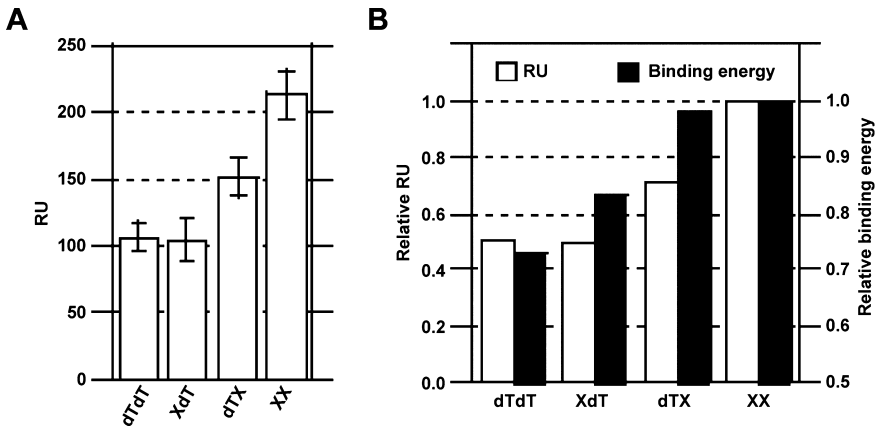

Figure 4. Binding assay of the modified siRNA strand with Ago2. (A) Surface plasmon resonance experiments were performed on a BIAcore X biosensor system to measure the affinity of the modified siRNA strand for Ago2. The experimental procedure is described in Experimental Procedures. (B) Binding energy value (shown in Table 1) for each modification and RU value (shown in panel A) of the corresponding strand normalized by maximizing the value to obtain the relative binding ability. Two sets of relative values for the chemical modification are plotted together.

Table 2. Summary of the Chemically Modified siRNAs[a]

	sense strand (SS) 3′-overhang (3′−5′)	antisense strand (AS) 3′-overhang (3′−5′)	inhibition (%)
siRNA1	dTdT	dTdT	54.48
siRNA2	XdT	dTdT	56.59
siRNA3	XdT	XX	62.32
siRNA4	XdT	dTX	75.04
siRNA5	XdT	XdT	64.81
siRNA6	dTX	XdT	61.5
siRNA7	dTX	dTX	64.11
siRNA8	X*dT	dTdT	68.54
siRNA9	X*dT	XX	62.48
siRNA10	X*dT	dTX*	76.38
siRNA11	X*dT	X*dT	66.72
siRNA12	dTX*	X*dT	59.51
siRNA13	dTX*	dTX*	66.86

[a]All siRNAs targeting luciferase contain the same duplex region: 5′-UGAAGAGCCUGAUCAAAUA-3′ and 3′-ACUUCUCGGACUAGUUUAU-5′. X* represents the derivative that has a 4-fluoro substituent on the benzene ring.

increase siRNA potency, e.g., siRNA4 and siRNA10. In contrast, siRNA6 and siRNA12 exhibited lower activity.

One of the major concerns is whether the improved effect of the modified siRNA results from nonspecific cytotoxicity. To address this question, we measured the cytotoxicity of siRNA4 and siRNA10, the two most potent siRNAs, in the SRB assay using Hep G2 cells. No inhibition of the growth of Hep G2 cells was observed in the presence of 100 nM modified siRNA (Figure 6), which was approximately 50 times higher than the concentration (2 nM) that produces gene silencing effects. It is worth noting that, compared to the unmodified siRNA, two modified siRNAs always show lower nonspecific toxicity at concentrations of up to 500 nM, which may result from a weaker off-target effect from the modified siRNAs.

Chemical Modification Improves the Ability of siRNA in Downregulation of MDM2 Expression and Antiproliferative Activity in MCF-7 Cells. To further validate our results and explore the potential of chemical modifications, we examined whether the XdT/dTX modification combination improves the interference activity of siRNA targeting other genes. In our previous study, we have identified a siRNA sequence that specifically inhibits Mdm2 expression.[26] The Mdm2 protein impairs the function of wild-type p53, Rb, and p21 and therefore stimulates the proliferation of the cells.[27] Both *in vivo* and *in vitro* results have shown that the Mdm2 siRNA significantly inhibits the proliferation of breast cancer.[26]

As shown in panels A and B of Figure 7, the siRNA with the XdT/dTX modification inhibited the expression of Mdm2 by >60% in tumor cell line MCF-7, a much greater knockdown efficiency ($p < 0.01$) compared with those of the unmodified and dTX/XdT-modified siRNAs. Consistent with the quantification results for the Mdm2 protein, Mdm2 mRNA was reduced to similar extents, e.g., 57, 48, and 31% of that of the control group when the cells were treated with the unmodified, dTX/XdT-modified, and XdT/dTX-modified siRNA, respectively (Figure 7C).

Next, we evaluated the antiproliferative activity of the modified siRNA in tumor cell line MCF-7 using a SRB method. As shown in Figure 7D, the growth of tumor cell MCF-7 was suppressed by the three siRNAs in a dose-dependent manner and the IC_{50} values for the unmodified, dTX/XdT-modified, and XdT/dTX-modified siRNA1 were approximately 100.6, 87.4, and 46.52 nM, respectively. These results suggest that the XdT/dTX modification significantly increases the antitumor activity, which was consistent with the efficient inhibition of MDM2 mRNA and protein expression.

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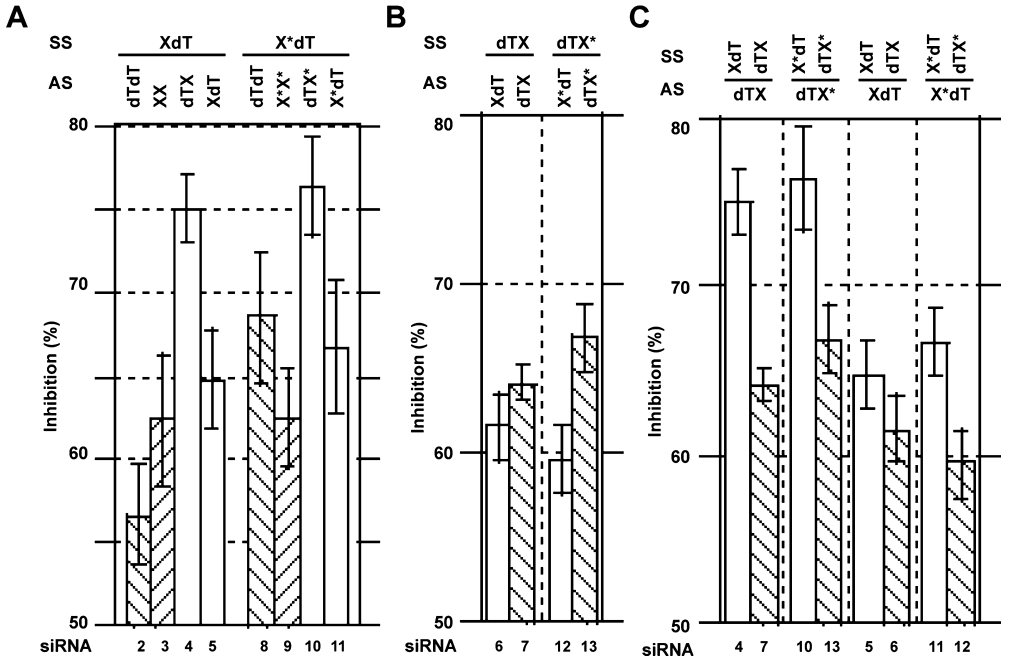

Figure 5. Activities of modified siRNA duplexes in a luciferase assay. The activities that were determined from cells transfected with the control siRNA were set at 100%. The means ± the standard deviation from three replicate experiments are presented.

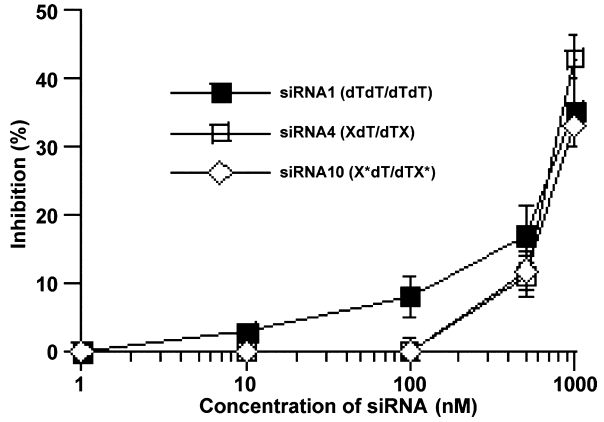

Figure 6. Inhibitory effect of modified siRNAs on the growth of Hep G2 cells. Hep G2 cells were transfected with various amounts of siRNAs. The growth of the cells was measured in a SRB assay. The experimental procedure is described in Experimental Procedures.

Chemical Modification Improves the Ability of siRNA To Deplete MOV10 Expression in HKE293 Cells. We next conducted experiments using siRNAs targeting the MOV10 gene to further validate our observations. Human cellular protein MOV10 has been reported to inhibit the replication of lentiviruses, including human immunodeficiency virus type 1 (HIV-1), via incorporation of MOV10 into HIV-1 particles and impeding viral reverse transcription.[28] Our recent report shows that MOV10 also restricts the mobilization of long interspersed element 1 (LINE-1), which is an autonomous retrotransposon.[20] As shown in panels A and B of Figure 8, the siRNA with the XdT/dTX modification downregulated MOV10 protein expression by 62% in cell line HEK 293, whereas only 34 and 40% knockdown of MOV10 protein was observed in the presence of unmodified siRNA and dTX/XdT-modified siRNA, respectively. Again, chemical modification XdT/dTX improved siRNA potency to knock down gene expression. This effect was also seen at the mRNA level (Figure 8C). Consistently, the greatest number of colonies representing LINE-1 transposition was found in the cells treated with the MOV10 siRNA containing the XdT/dTX modification (Figure 8D,E). These results further support the idea that the XdT/dTX modification, leading to higher binding affinity of the guiding strand and a lower binding affinity of the nonguiding strand for Ago2, improves siRNA potency.

DISCUSSION

In RNAi research, siRNAs are often designed with dTdT overhangs, but UU or UG overhangs are also common. Several studies demonstrated that siRNAs with different 3′-terminal dinucleotide overhangs have been shown to effectively induce RNAi. In this paper, we designed a set of modified 2 nt 3′-overhangs and evaluated their binding affinities for the PAZ domain through computer modeling and the *in vitro* SPR assay. The results revealed that increasing the binding affinity of the 3′-end of the guiding strand with Ago2 and/or reducing the binding affinity of the sense strand by the modified 2 nt 3′-overhangs significantly improves RNA interference activity. Clearly, this work provides, for the first time, a strategy for optimizing siRNA performance through asymmetric chemical modification of 3′-overhangs. A similar study reported a method using computational screening to identify functional 5′-end modifiers for siRNA guide strands.[29] These studies collectively indicate that the computer-aided design method is useful and predictive in designing the novel chemically modified monomeric siRNAs.

Molecular modeling predicts that modification at the second nucleotide (dTX and XX) enhances the interaction between the siRNA strand and the PAZ domain of Ago2, compared with those found with dTdT and XdT. The linked phosphate groups at the position 2-modified group retain the ribose five-ring structure with the linked phosphate groups, thus ensuring that the motif binds with the groove through the formation of hydrogen bonds. In addition, the pyrimidine structure at

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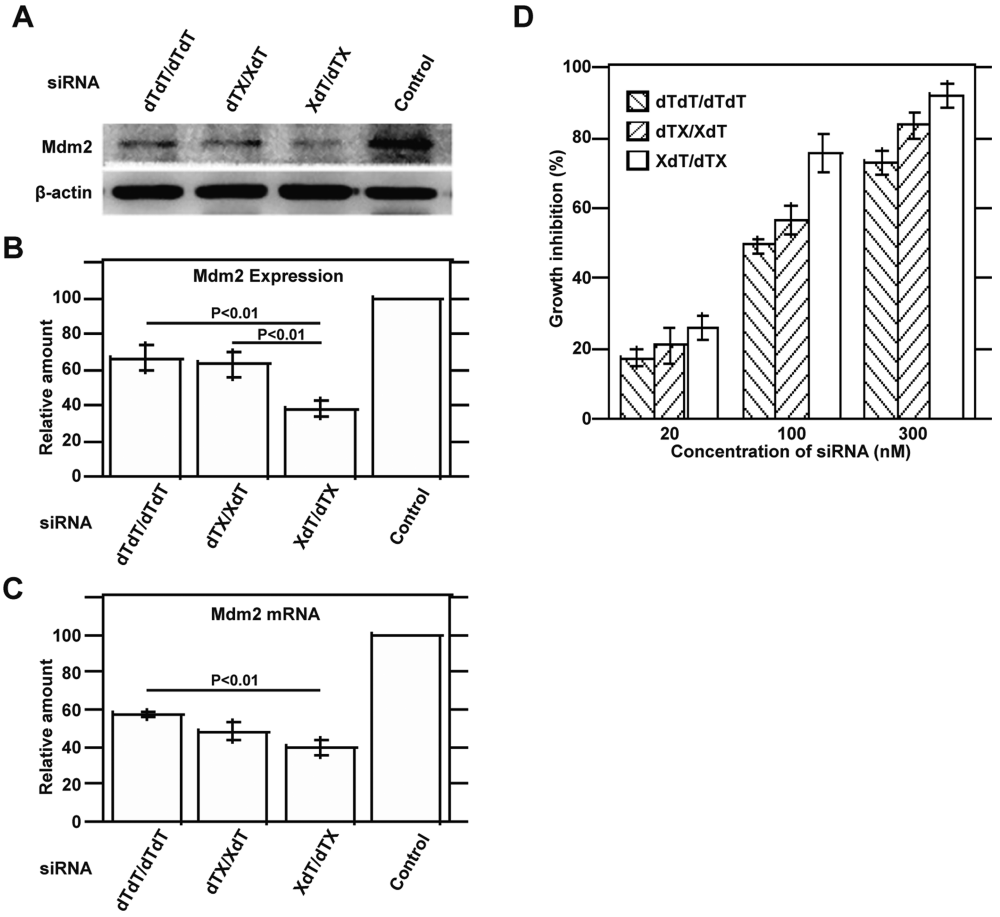

Figure 7. Activity of the modified siRNA in downregulation of Mdm2 expression and antiproliferative activity in MCF-7 cells. MCF-7 cells were transfected with either native or modified siRNAs. (A) Western blots to measure levels of endogenous Mdm2. (B) Results of three independent transfection experiments shown in panel A quantified and summarized. (C) Effect of siRNAs on Mdm2 mRNA expression measured by a real-time RT-PCR. (D) Effect of siRNA on proliferation of MCF-7 cells determined in a SRB assay. The activities that were determined for cells transfected with the control siRNA were set to 100%. The means ± the standard deviation from three replicate experiments are presented. The *p* values were calculated with a Student's *t* test.

position 2 was replaced with the hydrophobic group. This modification enhances the hydrophobic interactions between the aromatic motifs and the surrounding hydrophobic residues. Indeed, the result of the surface plasmon resonance (SPR) assay, which was used to measure the binding affinity of the modified siRNAs for Ago2 *in vitro*, validated the prediction of the *in silico* analysis.

The major goal of optimizing siRNA performance through chemical modification is to improve siRNA potency and reduce its off-target effects. Selective introduction of the guiding strand into the RISC is an essential step for improving RNA interference activity and avoiding off-target effects. Recent studies demonstrated that terminal structures of siRNA are involved in strand selection,[14−16] suggesting that chemical modifications of the 3′-overhang may enhance selection of the guiding strand. Indeed, our data show that the dTX modification of the antisense strand always improved the potency (Figure 4A) by strengthening the interaction between the 3′-end of the antisense strand and the PAZ domain. In contrast, the sense strand with stronger binding to Ago2 impairs the RNA interference effect (Figure 4C). We therefore conclude that a higher binding affinity of the guiding strand and a lower binding affinity of the nonguiding strand for Ago2 improve siRNA potency and thereby enhance RNA interference activity and reduce off-target effects. Indeed, in the experiments in which endogenous Mdm2 and MOV10 were knocked down using different modified siRNAs, the XdT/dTX modifications, exhibiting a higher binding affinity of the guiding strand and a lower binding affinity of the nonguiding strand for Ago2, showed the highest potency compared with those of other siRNAs containing different modifications (Figures 7 and 8).

It is also worth noting that the RNA strands with the XX type of 3′-end showed great binding affinity for Ago2 *in vitro*, yet this XX type of 3′-end in the antisense strand did not result in better potency as seen with dTX. The mechanism underlying the conflict remains unclear. One explanation is that the aromatic hydrophobic structure of the modified first nucleotide may affect the rearrangement of the conformation of the PAZ domain, which was shown to be critical for the catalytic cycle of Ago2 and the RISC.[30] Indeed, NMR binding studies of the PAZ domain with siRNAs showed the first overhanging nucleotide has a rather fixed position whereas the second overhanging nucleotide has greater flexibility.[17] Although the function of the fixed position of the first nucleotide is still unknown, introducing an aromatic hydrophobic group may cause a change in the position of the first nucleotide and thereby impair RNA interference.

In addition to the interaction between the PAZ domain and the 3′ end of guide RNA, binding of the AS 5′-end to MID of

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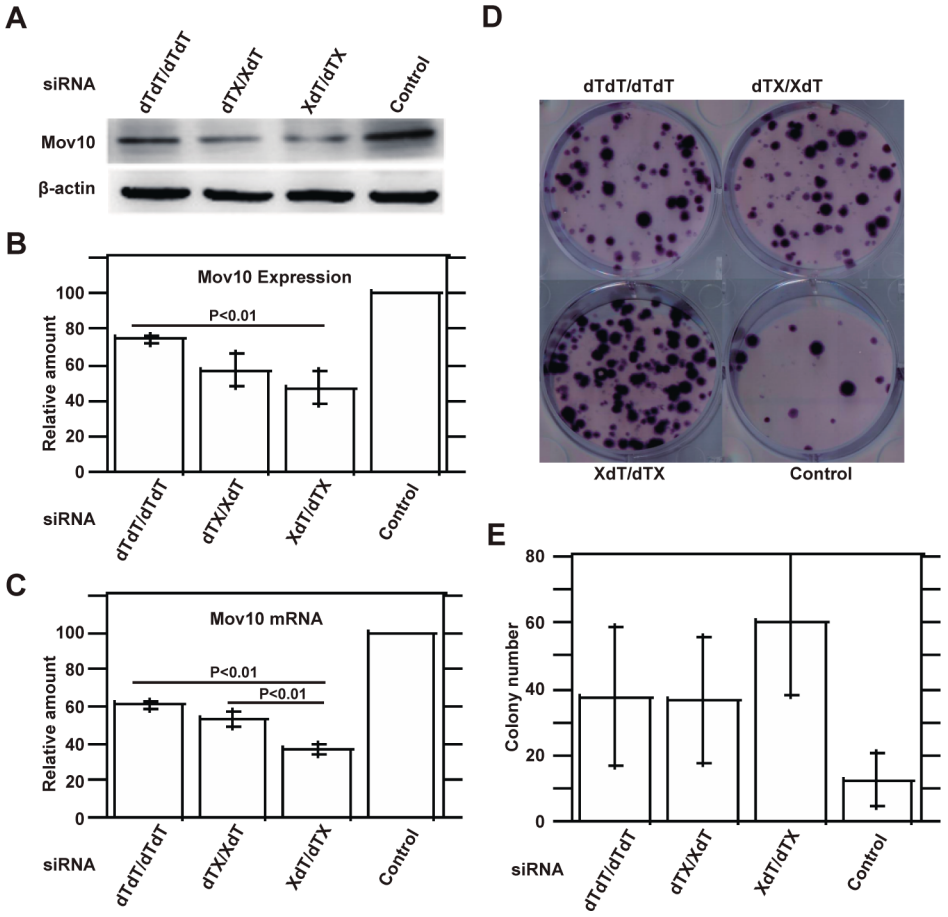

Figure 8. Activity of the modified siRNA in downregulation of MOV10 expression. (A) Western blots to measure levels of endogenous MOV10 in HEK 293 cells that were transfected with control siRNA or the modified siRNAs targeting MOV10 mRNA. (B) Results of three independent transfection experiments shown in panel A quantified and summarized. (C) Effect of siRNAs on MOV10 mRNA expression measured by real-time RT-PCR. (D) HEK 293 cells transfected with siRNA oligos prior to transfection with 750 ng of the CMV-L1-neoRT. The G418-resistant cell colonies were scored for each transfection. (E) Summary of the results of three independent transfection experiments shown in panel D. The means ± the standard deviation from three replicate experiments are presented. The *p* values were calculated with a Student's *t* test.

Ago2 also contributes to gene silencing efficiency. In our study, results in panels A and B of Figure 5 showed that, when the 3′-overhangs of sense strands have the same modification, the dTX modification of the antisense strand always generates the greatest inhibitory effect. This suggests the interaction between the 3′-end of the antisense strand and the PAZ domain rather than the recognition of the AS 5′-end by the MID domain leads to the improved siRNA potency, yet we cannot exclude the possibility that the modifications at the 3′-end of SS may also affect the recognition of the AS 5′-end by the MID domain. Future work is required to test this latter possibility.

In addition, we have designed and synthesized a novel series of chemically modified nucleotide monomers containing aromatic hydrophobic structure. These modified monomers contain both a straight chain and a benzene ring, which replace the pentose ring and the pyrimidine ring, respectively (Figure 2). The results of gene knockdown experiments revealed that the siRNA containing the modified nucleotides in 3′-overhangs elicits an RNA interference effect stronger than that of and a cytotoxicity lower than that of the native form. Our study provides the first evidence demonstrating the potential of the series of chemically modified nucleotide monomers in optimizing siRNA performance.

In summary, using computer modeling, we have successfully calculated the binding energy of siRNA with Ago2 and utilized the results in the design, prediction, and bioactivity screening for the novel chemically modified monomer. The results of biochemical assays and cell-based gene knockdown experiments show that this computer modeling-based strategy is feasible and predictive in designing the novel chemically modified monomer of siRNAs. Altogether, our work provides a methodology for optimizing siRNA performance through asymmetric chemical modification of 3′-overhangs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of the chemically modified X monomer and X* monomer. 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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