Site-Specific Modification Using the 2′-Methoxyethyl Group Improves the Specificity and Activity of siRNAs

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Rapid progress has been made toward small interfering RNA (siRNA)-based therapy for human disorders, but rationally optimizing siRNAs for high specificity and potent silencing remains a challenge. In this study, we explored the effect of chemical modification at the cleavage site of siRNAs. We found that modifications at positions 9 and 10 markedly reduced the silencing potency of the unmodified strand of siRNAs but were well tolerated by the modified strand. Intriguingly, addition of the 2′-methoxyethyl (MOE) group at the cleavage site improved both the specificity and silencing activity of siRNAs by facilitating the oriented RNA-induced silencing complex (RISC) loading of the modified strand. Furthermore, we combined MOE modifications at positions 9 and 10 of one strand together with 2′-O-methylation (OMe) at position 14 of the other strand and found a synergistic effect that improved the specificity of siRNAs. The surprisingly beneficial effect of the combined modification was validated using siRNA-targeting endogenous gene intercellular adhesion molecule 1 (*ICAM1*). We found that the combined modifications eliminated its off-target effects. In conclusion, we established effective strategies to optimize siRNAs using site-specific MOE modifications. The findings may allow the creation of superior siRNAs for therapy in terms of activity and specificity.

INTRODUCTION

Small interfering RNAs (siRNAs) are 19–22 nt double-stranded RNA duplexes that have become a powerful tool for silencing the expression of targeted genes. After delivery into cells, the 5′ end of siRNA is phosphorylated by Clp1 kinase[1] and is recognized by TAR RNA-binding protein (TRBP) and Dicer for loading into the AGO2 protein, to assemble as the RNA-induced silencing complex (RISC).[2] It is known that RISC assembly is asymmetric: one strand (the guide or antisense strand) of the siRNA is retained in the complex, while the other (the passenger or sense strand) is normally cleaved at a position located 10 nt from the 5′ end of the guide strand and is discarded.[3] The mature RISC subsequently binds and cleaves the intended target RNAs with a perfect sequence complementary to the guide strand, resulting in sequence-specific gene silencing.

The application of siRNA drugs holds enormous potential in therapeutics. Much effort has been made to introduce siRNAs for the treatment of diseases for which no effective intervention exists.[4–6] However, many difficulties need to be overcome before such a goal can be realized. The specificity and silencing activity of siRNA are the most important issues in clinical and non-clinical applications.[7] Although a siRNA is designed to be perfectly complementary to the sequence of a target mRNA, genome-wide analysis has demonstrated that extensive other mRNAs are downregulated.[8] The off-target effects of siRNA are generally derived either from the guide strand via imperfectly matched sites or from interaction of the passenger strand with complementary transcripts other than the target mRNAs.[9–11]

The guide strand is determined before the two strands of an siRNA enter into AGO2. It has been proposed that the strand preferred by the RISC depends mainly on thermodynamic differences between siRNA duplexes.[12] Our results and those of others have demonstrated that both strands of a siRNA appear to have some probability of entering the RISC and triggering the RNAi process, though this varies from siRNA to siRNA.[13,14] Therefore, facilitating guide-strand selection is important for the specificity and potency of a siRNA and has been demonstrated to be an effective approach to improving siRNA efficacy and reduce passenger strand-mediated off-target effects.[13,15–17]

A variety of chemical modifications are documented to influence the strand selection of siRNAs, minimize the incorporation of the passenger strand, and thereby reduce their off-target effects.[13,15–17] Extensive incorporation of chemical groups at
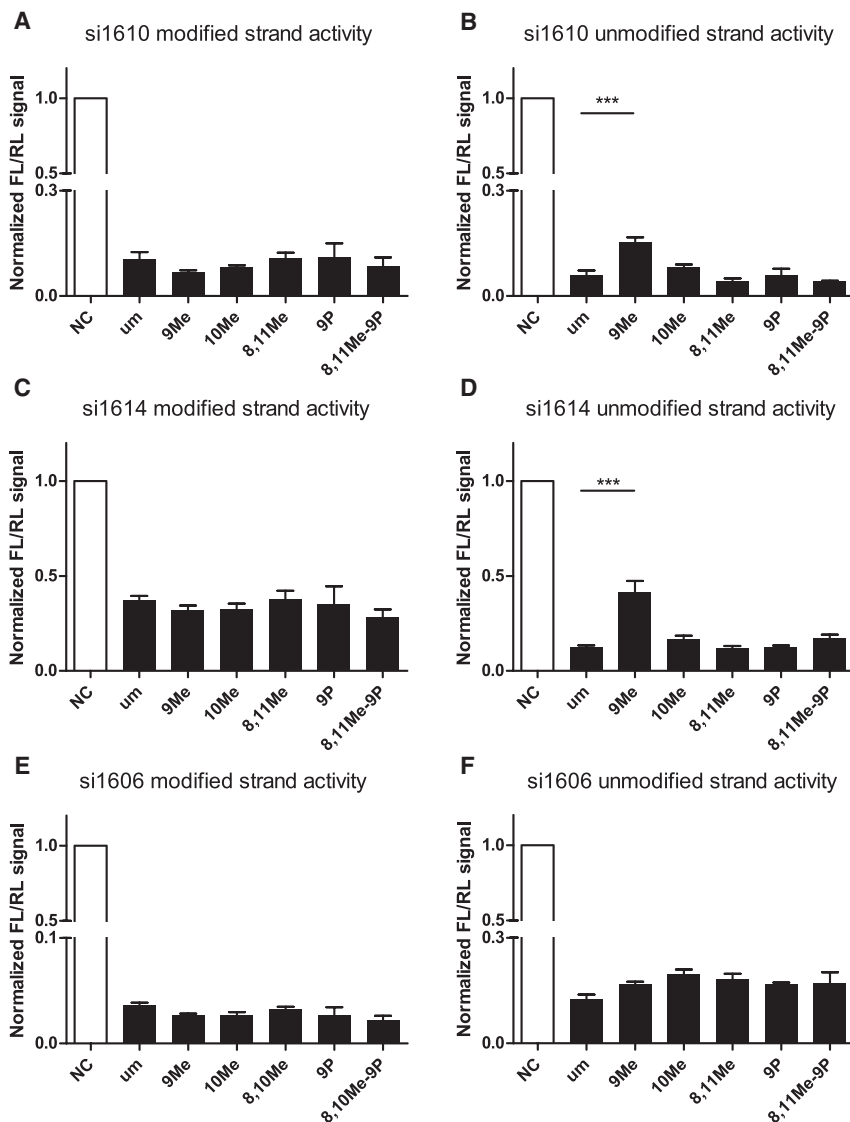
Figure 1. Effects of Chemical Modifications at the Scissile Sites of siRNA Strands on Silencing Activity

The silencing activity of modified and unmodified strands of siRNA si1610 (A and B), si1614 (C and D), and si1606 (E and F) quantified using dual-luciferase assays after site-specific modifications. Mean ± SEM; n = 3; ***p < 0.0001 compared with unmodified siRNAs. Except for marked pairs, there was no difference compared with the unmodified siRNA. FL, firefly luciferase; RL, *Renilla* luciferase.

site,[3] resulting in inefficient cleavage and impaired silencing of target RNA. We hypothesized that modifications at cleavage sites of siRNA may affect preferred strand loading to the RISC and silencing activity. In this study, we chose small RNAs with equivalent activity in both strands to investigate the effects of cleavage site-specific modifications on the efficacy and specificity of siRNAs.

RESULTS

Effects of Cleavage Site-Specific Modifications on Silencing Potency of siRNA Strands

To determine the effects of chemical modifications on siRNA strands, we tested the strand activity of siRNAs modified with OMe and PS at or near the scissile phosphodiester bond. Three siRNAs, si1610, si1606, and si1614, both strands of which were effectively assembled into the RISC complex and had efficient RNAi effects, were chosen from our previous study.[24] The sequences and chemical modification of these siRNAs are shown in Supplemental Materials and Methods. The OMe was added at positions 8, 9, 10, and 11, and the PS modification was placed between positions 9 and 10 of the guide strand. The gene-silencing activity of both strands of these modified siRNAs was quantified using dual-luciferase assays.

We found that all the site-specific modifications were well tolerated by the modified strands of all three siRNAs without affecting their activity (Figures 1A, 1C, and 1E), but OMe showed different effects on the unmodified strands. The OMe at position 9 compromised the gene silencing of the unmodified strand of si1610 and si1614 (Figures 1B and 1D). We assumed that addition of OMe at scissile sites might impair the cleavage or removal of the modified strand, which in turn reduced the silencing potency of the unmodified strand. No significant effect was found for the unmodified strand of si1606 (Figure 1F), suggesting that the effect of OMe at position 9 was influenced by specific sequences.
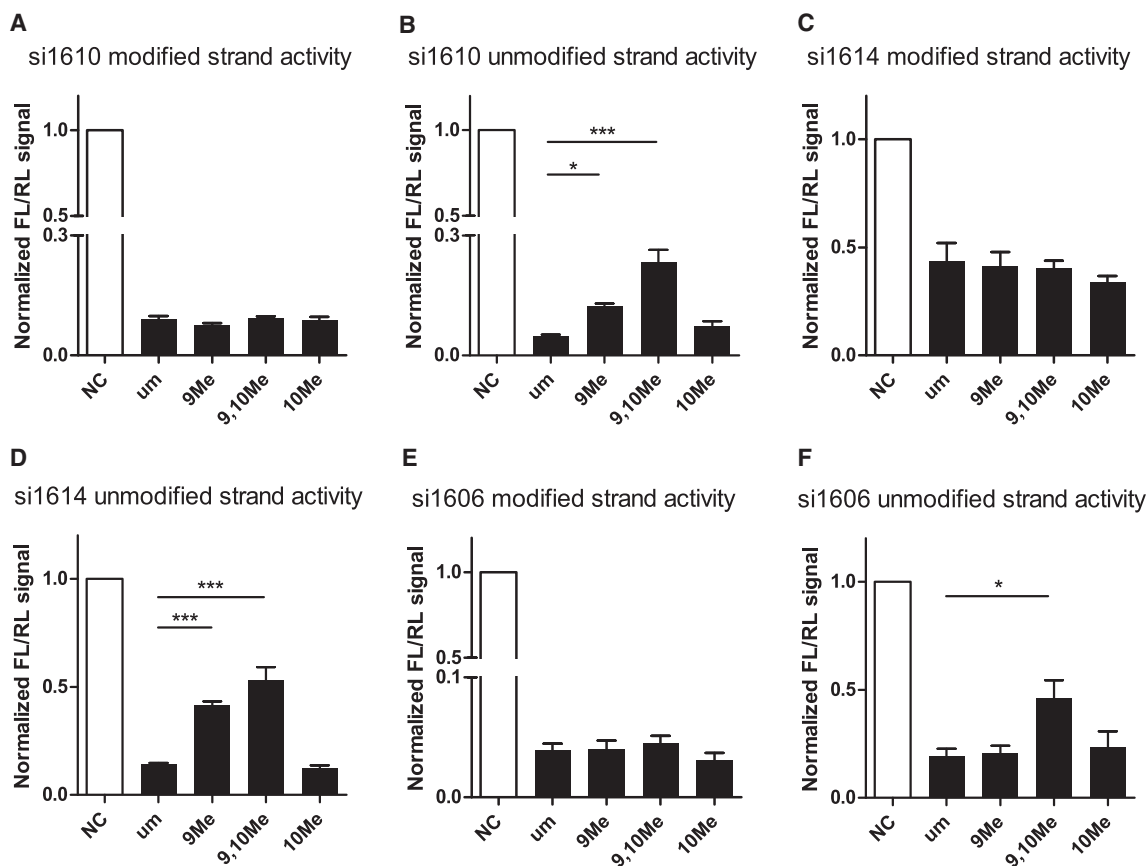
multiple sites entails a relatively large cost, while importantly, it may reduce siRNA activity[10,18,19] or cause cytotoxicity.[20] Therefore, modifications at specific positions in siRNAs are desirable. Several studies, including ours, have shown improved effects on the specificity and activity of siRNAs by site-specific modifications, such as unlocked nucleic acid (UNA), locked nucleic acid (LNA), and 2′-O-methylation (OMe).[10,14,16,18,21,22] In a previous study, we found that 2′-OMe at position 14 reduces RISC loading and the off-target effects of the modified strand.[23] Despite these efforts, more effective and applicable modification strategies for siRNA design are needed.

The passenger strand is highly complementary to the guide strand and can be cleaved as a target of the guide strand. It has been shown that cleavage of the passenger strand can be blocked by OMe or a phosphorothioate (PS) modification at this scissile

Figure 2. Combined OMe Modifications at Positions 9 and 10 Suppressed the Silencing Effect of the Unmodified Strand

The silencing activity of modified and modified strands of siRNA si1610 (A and B), si1614 (C and D), and si1606 (E and F) quantified by dual-luciferase assays. Mean ± SEM; n = 3; *p < 0.05 and ***p < 0.0001 compared with unmodified siRNAs. Except for marked pairs, there was no difference compared with the unmodified siRNA.

Combined OMe at Positions 9 and 10 Suppressed the Silencing Effect of the Unmodified Strand

Next, we investigated the effects of combined OMe at positions 9 and 10 of the siRNA strands by measuring their activity and found that for all the selected siRNAs, even when positions 9 and 10 were modified simultaneously, the potency of the modified strands was still unaffected (Figures 2A, 2C, and 2E). However, the activity of unmodified strands, including that of si1606, was significantly impaired by strand-specific modification compared with the unmodified siRNA control (Figures 2B, 2D, and 2F). Intriguingly, even if a single modification did not have a significant effect on the activity of the unmodified strand, the combined modifications at positions 9 and 10 had a much better effect. This suggested that the combined modifications had a synergistic effect.

Moreover, we modified the passenger strand of si1610 to determine the effect of OMe at positions 9 and 10 and found that this modification provided results consistent with those for the guide strand (Figure S1). Therefore, we concluded that site-specific OMe at positions 9 and 10 of one strand of siRNA affected the silencing activity of the unmodified strand without impairing the modified strand.

Effects of Different Modifications at Positions 9 and 10 on the Silencing Activity of siRNA Strands

To further confirm the effect of combined modifications at positions 9 and 10, we chose two siRNAs, si1610 and si1606, and modified their guide strands with another two commonly used chemical groups, the fluorine (F) and 2′-methoxyethyl (MOE) groups. Consistent with the results of OMe, placement of both F and MOE modifications did not block the silencing activity of the modified strands (Figures 3A and 3C). However, the activity of unmodified strands was interrupted to varying degrees. For modification with the bulky MOE group, a much more compromised effect was found than with OMe (Figures 3B and 3D). Even a single MOE at position 9 or 10 dramatically impaired the silencing activity of the unmodified strand. When both positions 9 and 10 had an MOE group, the activity of the unmodified strand was further reduced. In contrast, 2′F modification of the guide strand did not impair the activity of the unmodified passenger strand (Figures 3B and 3D). We speculated that the effects of modification depended largely on the size of the modifying chemical group (F < OMe < MOE).

To validate whether it is a general rule that the silencing potency of the unmodified siRNA strand is susceptible to MOE modification at
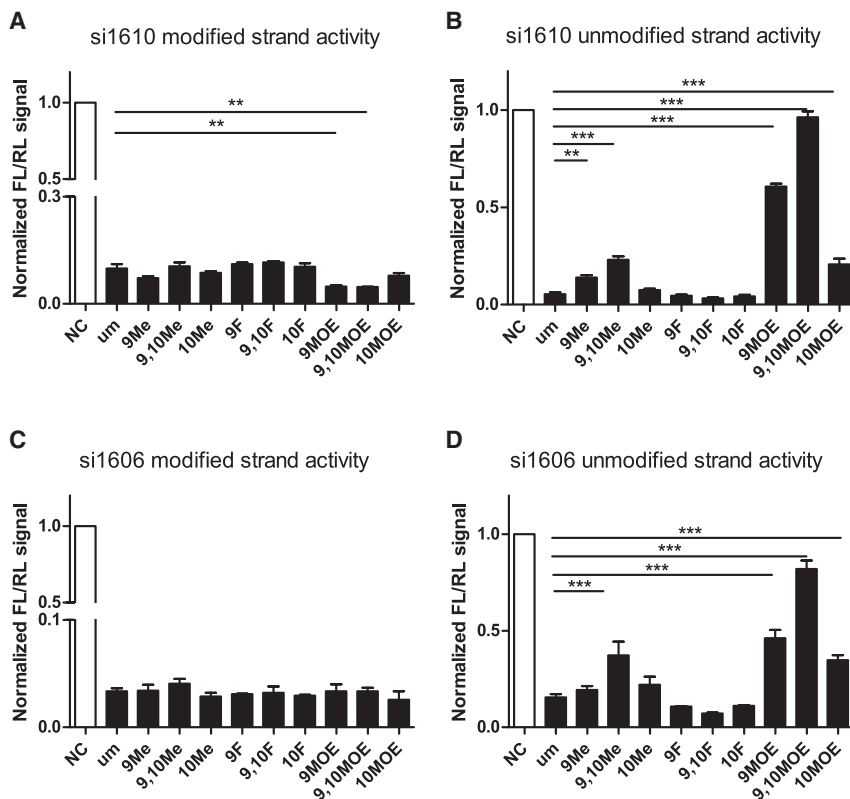
Figure 3. Effects of Different Modifications at Both Positions 9 and 10 of One Strand of siRNA on Silencing Activity

The silencing activity of modified and unmodified strands of siRNA si1610 (A and B) and si1606 (C and D) quantified by dual-luciferase assays after diverse modifications. Mean ± SEM; n = 3; **p < 0.01 and ***p < 0.0001 compared with unmodified siRNAs.

MOE Modification at Positions 9 and 10 Improves the Silencing Potency of the Modified Strand

The unexpected finding that MOE modification increased the RISC loading of the modified strand indicated that the silencing activity of this strand may be improved. We did note the enhanced silencing potency of the MOE-modified strands of si1610 and siCdc2, but this effect was not found for siRNAs 1606 and NYP305 (Figures 3A, 3C, 4A, and 4C). We re-analyzed the data and considered that the gene-silencing potency of the guide strands of siRNA 1606 and NYP305 was too strong at 16.7 nM, masking the improvement due to MOE modification. Hence, to verify this speculation, we diluted these siRNAs to 0.167 nM and then evaluated the silencing efficiency of the modified strand. As expected, we found that the silencing potency was improved (Figures 6A and 6B).

Furthermore, we used another two siRNAs, simir-1 and siGAPDH,[25] which have moderate silencing potency (remaining activity ≥ 50%), and determined the potency of the MOE-modified strands. We found that the silencing efficacy of the modified strand was improved significantly with MOE modification at positions 9 and 10 (Figures 6C and 6D). Therefore, we concluded that MOE modifications at positions 9 and 10 provide an applicable strategy to improve the power of siRNAs with moderate silencing activity.

Combination of MOE at Positions 9 and 10 with OMe at Position 14 Diminished or Eliminated Off-Target Effects

The MOE modification at positions 9 and 10 regulated strand selection in RISC loading, which might be used to eliminate the off-target effects of siRNA. We previously identified that strand-specific 2′-OMe at position 14 reduces the off-target effects of the modified strand.[23] We thereby hypothesized that combining OMe modification at position 14 with MOE modification at positions 9 and 10 would have a better effect on reducing off-target effects and chose siCdc2 for the test. When the guide strand was modified with MOE at positions 9 and 10, we found that these modifications had no effect on the guide strand activity (Figure 7A), but the silencing potency of the passenger strand was reduced. More important, the target repression of the passenger strand was further reduced when this strand was simultaneously

positions 9 and 10, we modified another two siRNAs, siCdc2 and siNYP305, which showed good silencing potency in both strands in our previous study.[23] We found that addition of MOE at both positions 9 and 10 had a strong inhibitory effect on the silencing activity of the unmodified strand (Figures 4A and 4B), while the activity of the modified strand was not disrupted (Figures 4C and 4D).

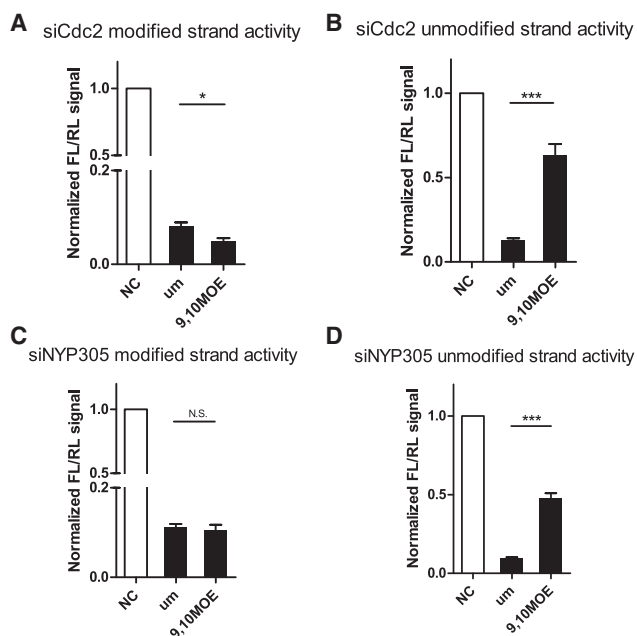## MOE Modification at Positions 9 and 10 Affects the RISC Loading of Both siRNA Strands

To explore the mechanism by which MOE modification at positions 9 and 10 affected the silencing activity of the siRNA strands, we determined the RISC-loading levels of the strands after modification using RNA immunoprecipitation (RIP). The unmodified native siRNA 1606-um and its modified counterpart 1606-9,10MOE were transfected into HEK293A cells. The RISC-loaded strand of siRNA was immunoprecipitated by anti-AGO2 and quantified using real-time PCR. We found that the RISC-loading level of the unmodified strand was markedly decreased after site-specific MOE modification (Figure 5A). The change of loading level was consistent with the reduced silencing potency in dual-luciferase assays (Figure 3D). Intriguingly, the MOE modification increased the RISC loading of the modified strand (Figure 5B). Taken together, MOE modification at positions 9 and 10 reduced the RISC loading of the unmodified strand and increased the loading of the modified strand.

Figure 4. MOE Modifications at Positions 9 and 10 of siRNA Alleviates Silencing Activity of the Unmodified Strand
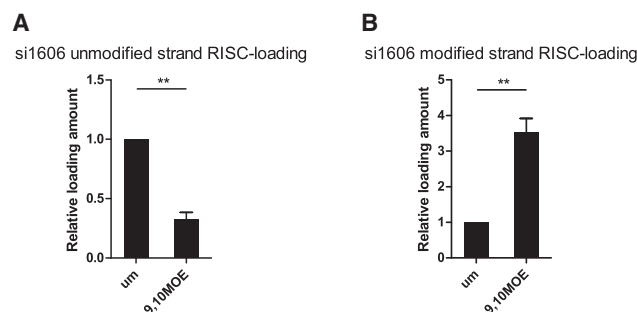
The silencing activity of modified and unmodified strands of siRNA siCdc2 (A and B) and siNPY305 (C and D) quantified by dual-luciferase assays after MOE modifications. Mean ± SEM; n = 3; *p < 0.05 and ***p < 0.0001 compared with unmodified siRNAs.

Figure 5. MOE Modifications at Positions 9 and 10 Affect the RISC Loading of Both Strands of siRNA

Unmodified siRNA 1606-um and its modified counterpart 1606-9,10MOE were transfected into HEK293A cells at a final concentration of 16.7 nM. RISC-loaded strands of siRNA were immunoprecipitated by anti-AGO2. Unmodified (A) and modified (B) strands of siRNA quantified by qPCR. Mean ± SEM; n = 4; **p < 0.01 compared with unmodified siRNAs.

modified with OMe at position 14 (Figure 7B). When the MOE was placed in the passenger strand with OMe in the guide strand, consistent results were obtained (Figures 7C and 7D). These findings indicated that the combination of MOE modification at positions 9 and 10 with OMe at position 14 can be used to reduce the off-target effects of siRNAs.

To further assess whether this strategy was applicable to an endogenous target, we examined the siRNA active against intercellular adhesion molecule 1 (*ICAM*), which has been shown to reduce *ICAM* expression (on-target) in human umbilical vein endothelial cells (HUVECs) but exhibits unintended knockdown of tumor necrosis factor receptor (*TNFR*) (off-target) via its passenger strand.[26] So we transfected unmodified siICAM-um and its modified counterparts into cultured HUVECs (Figure 8A). The silencing of on-target and off-target genes was evaluated by qPCR. Consistent with our earlier results, the MOE at positions 9 and 10 reduced the off-target silencing of *TNFR*. When the MOE on the guide strand was combined with OMe at position 14 on the passenger strand, the off-target silencing was almost completely eliminated (Figure 8B). Of note, the MOE or combined MOE/OMe modification enhanced the silencing effect on the designated target *ICAM* (Figure 8C).

The siRNAs with Combined Chemical Modifications Did Not Show Cytotoxicity

To evaluate the toxicity of siRNAs modified with chemical modifications, we determined the possible cytotoxicity of the unmodified

siRNA and the modified counterparts using MTT assays. Cells transfected with OMe and/or MOE modified siRNAs, and no difference in cell toxicity was found between siRNA and mock transfection groups (Figure S2). This characteristic laid the foundation for the application in future studies.

In addition, to find out whether the increased or decreased activity of strands in our study resulted from changed transfection efficiency, we transfected the Cy5-labeled siRNAs with or without modification. We found that they had similar transfection efficiency (Figure S3).

DISCUSSION

In the present study, we identified novel site-specific chemical modifications to control guide-strand selection by the RISC and the activity of siRNAs. The MOE modifications at positions 9 and 10 of one strand reduced the activity of the unmodified strand, while facilitating gene-silencing activity of the modified strand by regulating the RISC loading. Moreover, combined modification at the cleavage site of the guide strand with modification at position 14 of the passenger strand synergistically abolished the off-target effect caused by the unmodified strand. Our findings provide an effective strategy for improving siRNA specificity and potency.

Preferential strand selection can be achieved by position-specific modifications.[13,15–17] The known modification approaches focus on either altering the thermodynamic stabilization of the siRNA duplex or disturbing the interactions between the ribonucleotide strands and the MID or PAZ domain of AGO2.[10,14,16,18,21,22,27] Here, we demonstrated an excellent effect of modification at the scissile site on the selection of siRNA strands, which provided a novel modification strategy to control strand selection by promoting oriented RISC loading.

MOE modification has been used to increase the target-binding affinity and improve the nuclease stability of siRNAs.[28] Previous studies have shown that the MOE group is well tolerated in the passenger strand but results in less active siRNA silencing activity of the guide
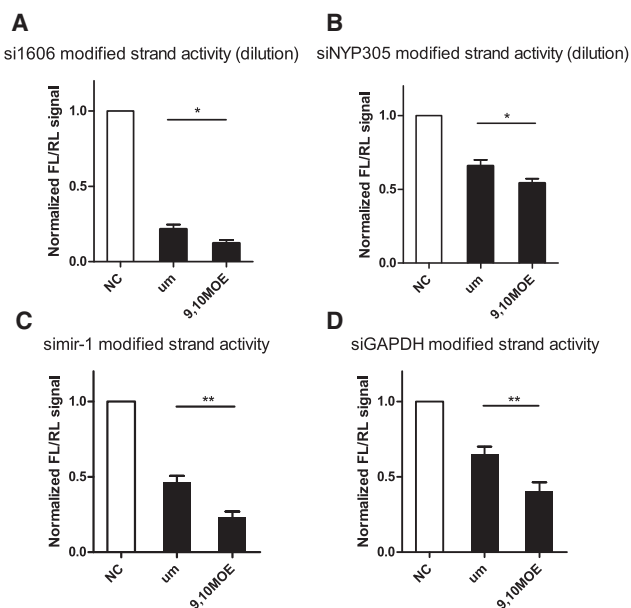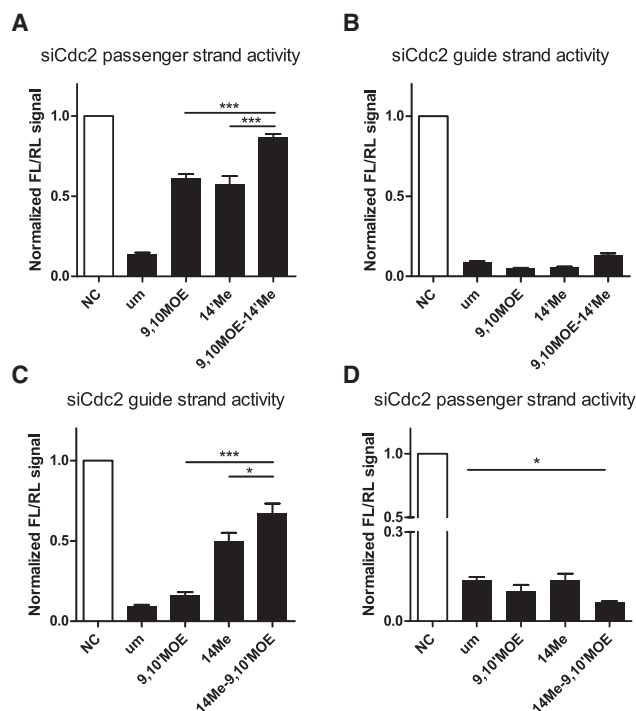
Figure 6. MOE Modifications at Positions 9 and 10 Improved the Silencing Potency of the Modified Strand

Modified strand activity of siRNA si1606 (A) and siNYP305 (B) quantified at a low concentration of 0.167 nM and siRNA simir-1 (C) and siGAPDH (D) quantified at 16.7 nM by dual-luciferase assays after MOE modifications. Mean ± SEM; n = 4; *p < 0.05 and **p < 0.01 compared with unmodified siRNAs.
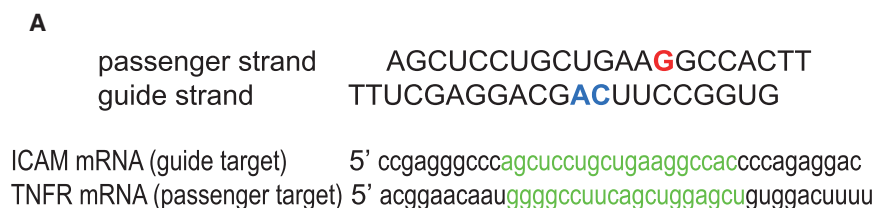

Figure 7. Combination of MOE with OMe Modifications Diminishes or Eliminates Off-Target Effects of siRNA

(A and B) The guide strand of siRNA Cdc2 was modified with MOE at positions 9 and 10, and the passenger strand was modified with 2′-OMe at position 14. The silencing activity of both strands was quantified by dual-luciferase assays. (C and D) The passenger strand of siRNA Cdc2 was modified with MOE at positions 9 and 10, and the guide strand was modified with 2′-OMe at position 14. The silencing activity of both strands was quantified as above. Mean ± SEM; n = 4; *p < 0.05 and ***p < 0.0001 compared with combined modified siRNAs.

strand regardless of its placement.[29] In this study, we unexpectedly found that MOE modifications incorporated into one strand at positions 9 and 10 not only reduced the activity of the unmodified strand but also improved the RISC loading and silencing activity of the modified strand. To our knowledge, this is the first evidence of site-specific MOE modification to enhance the on-target silencing activity of the modified strand. Particularly, in many real cases, siRNAs with moderate knockdown activity must be used because the number of siRNA candidates for the target RNA is limited by the short alternative sequences (e.g., SNPs) and species-specific or isoform-specific sequences.[14,30,31] In these cases, MOE modification at positions 9 and 10 provides an applicable strategy to improve their silencing activity. Of note, 2′-fluoro modification did not reduce the silencing activity for unmodified strand. Previous data show that 2′F modification enhances Ago protein cleavage rate, while OMe abrogates cleavage at the same position,[32] and the electronegative 2′F group is considered to stabilize the developing negative charge of the 3′ oxygen-leaving group during the transition state.

Combining different modifications of siRNAs may integrate different beneficial properties and generate complementary or synergistic effects. We previously found that modification at position 14 could be used to diminish the off-target effects of siRNA effectively.[23] Here, we combined OMe modification at position 14 of one strand with MOE modification at positions 9 and 10 of the other strand and found that the combined modification achieved surprisingly good effects on the specificity and potency of the selected siRNAs.
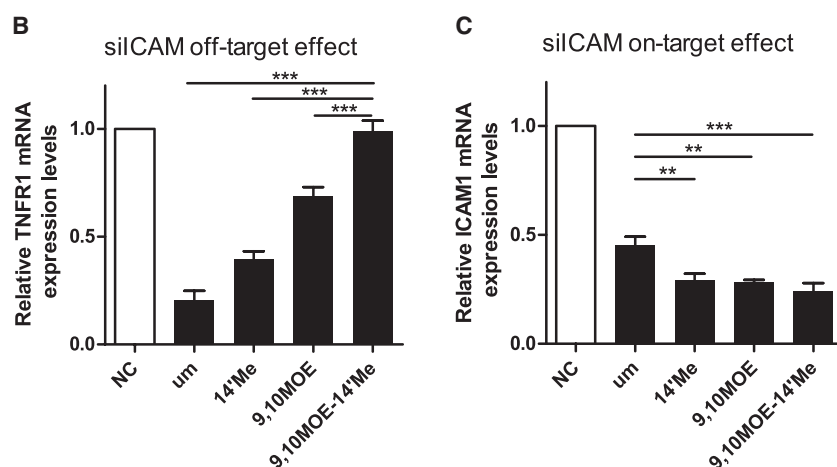
We speculated that the combined modification could have a synergistic effect on guide-strand selection. Cleavage of the guide strand might be impaired by the modifications at positions 9 and 10, and the interaction between the passenger strand and AGO protein is disrupted by the modification at position 14, which synergistically facilities RISC loading of the guide strand. In brief, our findings indicate the importance of these strand-specific sites in the RNAi process, and combination of position-specific modifications is suggested as a potent strategy for reducing off-target effects triggered by the passenger strand.

Another interesting finding in this study was that the addition of MOE at positions 9 and 10 increased the RISC loading of the modified strand while decreasing the loading of the unmodified strand. This led us to speculate that the RISC entry of siRNA is reversible. That is, there are two forms of siRNA duplex with different guide strands to be recognized by TRBP and Dicer after modification. When the unmodified strand acts as the guide strand, the siRNA is less potent because of the impairment of passenger strand cleavage by the modification. On the other hand, when the modified strand performs as the guide strand, the siRNA is potent. That is, these two forms of siRNA compete.[33] When cleavage of the passenger strand is impaired

A

passenger strand	AGCUCCUGCUGAA**G**GCCACTT
guide strand	TTUCGAGGACGA**AC**UUCCGGUG

ICAM mRNA (guide target)   5' ccgaggggccc agcuccugcugaaggccac cccagaggac

TNFR mRNA (passenger target)   5' acggaacaau ggggccuucagcuggagcu guggacuuuu

Figure 8. Combined MOE and OMe Modification Eliminates Off-Target Effect of siRNA for an Endogenous Gene

(A) Sequence of siRNA for *ICAM* and its endogenous target genes. The target sequences in the gene transcripts and the modified positions are underlined. (B and C) Knockdown of *ICAM* and *TNFR* expression in HUVECs using different modified siRNAs. Mean ± SEM; n = 5; ***p < 0.0001 compared with combined modified siRNAs.

B

siICAM off-target effect

C

siICAM on-target effect

siQuant vector. The short fragment is ligated with siQuant vector. Thus a complete fusion gene including both the siRNA target site and intact firefly luciferase in the correct read frame is constructed. The expression change of the transcript is used to determine the siRNA silencing efficacy.

Cell Cultures

HEK293 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM l-glutamine, 100 U/mL penicillin, and 100 g/mL streptomycin (GIBCO).

HUVECs were cultured in Endothelial Cell Medium (ScienCell) supplemented with 5% (v/v) FBS, 1% Endothelial Cell Growth Supplement, and 1% penicillin/streptomycin. HUVECs within three to seven passages were used.

because of modification, the siRNA duplex cannot remain stable in the AGO2 protein and is then squeezed out. The strands have opportunities to re-enter, for they both have the potential to be assembled efficiently into the RISC. Taken together, our data suggest that the RISC entry of siRNA can be reversible, though direct evidence is needed from future studies.

In conclusion, our study is an example of a successful combined modification strategy to eliminate off-target effects. The principles of the site-specific modifications established in this study allow the creation of superior siRNAs in terms of activity and specificity. We provide a novel strategy, which may be particularly important for siRNAs with equal potency in both strands and to optimize siRNAs for animal studies or therapeutic applications.

MATERIALS AND METHODS
Oligonucleotide Preparation and Plasmid Constructs

DNA oligonucleotides were from Biosune, and siRNAs were from Ribobio. The siRNA sequences used are listed in Supplemental Materials and Methods.

The sequence-dependent silencing effect of siRNA was validated in our previous study using the siQuant system. The reporter plasmids were constructed by inserting target sequences complementary to the siRNA strands into the siQuant reporter plasmids.[34] Sense and antisense DNA oligonucleotides corresponding to the selected siRNA target site are annealed to form a short duplex with complementary ends to linearized

Dual-Luciferase Assay

HEK293 cells were plated into 24-well plates at $\sim 1 \times 10^5$ cells/well 24 hr before transfection. siQuant vector (100 ng/well) carrying the target site of the tested siRNA was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) at ~70% confluence, together with the pRL-TK control vector (50 ng/well) and the siRNA at the appropriate concentration. The activity of both luciferases was determined 24 hr after transfection using a Synergy HT fluorometer (BioTek Instruments). Cells were lysed with Passive Lysis Buffer (Promega). Cell lysate (10 μL) was transferred into a 96-well plate, and the substrate reagents were added. Firefly and *Renilla* luciferase activity was evaluated using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions, and the firefly activity was normalized to the *Renilla* activity. All experiments were performed in duplicate and repeated at least three times.

RIP Assay

For RIP, HEK293 cells were grown in 10 cm² dishes and transfected with unmodified or modified siRNA 1606 using Lipofectamine 2000 at a final concentration of 16.7 nM. Protein A beads (Invitrogen) were pre-swelled for 2 hr at 4°C with NT2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM MgCl₂, and 0.05% Nonidet P-40) supplemented with 5% BSA. After pulse centrifugation (400 × *g* for 30 s), the supernatant was discarded and substituted by ice-cold NT2 buffer

and 4 μg antibody, AGO2 antibody (Abnova), or mouse IgG (Invitrogen). After incubation overnight with rotation, the antibody-coated beads were washed four times with ice-cold NT2 buffer and re-suspended in 800 μL NT2 buffer with 200 U RNase inhibitor, 400 μM vanadyl ribonucleoside complexes, 1 mM DTT, and 20 mM EDTA. Cells were harvested 24 hr after transfection and crosslinked using 1% formaldehyde for 10 min at room temperature. Then glycine was added to a final concentration of 125 nM to quench the crosslinking. The cells were lysed with polysome lysis buffer (10 mM HEPES [pH 7.0], 100 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, and 0.5% Nonidet P-40) on ice for 0.5 hr. After centrifugation for 15 min at 15,000 × g, the mRNP supernatant was transferred to a prepared bead slurry and rotated for 6 hr at 4°C. After 6 hr of incubation, the beads were washed with ice-cold NT2 buffer, and the supernatant was removed. The beads containing the immunoprecipitated samples were collected and re-suspended in 100 μL Proteinase K buffer (100 mM NaCl, 10 mM Tris-Cl [pH 7.0], 1 mM EDTA, and 0.5% SDS) with 5 μL Proteinase K and incubated at 50°C for 45 min to reverse the crosslinks. The RNA from the immunoprecipitated pellet was isolated using TRI reagent (Invitrogen) and then reverse-transcribed into cDNA using a bulge-loop microRNA (miRNA) qRT-PCR primer set (Ribobio). The single-strand RNA level was measured using a qRT-PCR assay.

RNA Isolation and qRT-PCR Assay
Unmodified or modified siRNA ICAM (50 nM) was transfected into cultured HUVECs using Lipofectamine 2000. After 24 hr, total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions and then reverse-transcribed into cDNA using Oligo(dT)$_{18}$ primer and TransScript reverse transcriptase (Transgene). The expression levels of ICAM or TNFR were measured using a qRT-PCR SYBR green kit (Tiangen) in a StepOne Plus System (Applied Biosystems), with GAPDH as the internal control. The primers used in the assay were *ICAM*-F, 5′AGC TTCTCCTGCTCTGCAAC; *ICAM*-R, 5′AATCCCTCTCGTCCAG TCG; *TNFR*-F, 5′GCCAGGAGAAACAGAACACC; *TNFR*-R, 5′CTC AATCTGGGGGTAGGCACA; *GAPDH*-F, 5′GCTCTCTGCTCCTCC TGTTC; and *GAPDH*-R, 5′ACGACCAAATCCGTTGACTC.

Reverse transcription conditions were as follows: 30 min at 42°C followed by 10 min at 85°C. qRT-PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 64°C, and 20 s at 72°C. The data were calculated using the ΔCt equation as follows:

$$\Delta\Delta Ct = \left[Ct_{(siRNA,target)} - Ct_{(siRNA,GAPDH)}\right] - \left[Ct_{(NC,target)} - Ct_{(NC,GAPDH)}\right],$$

and the relative mRNA level corresponded to the $2^{-\Delta\Delta Ct}$.

Cell Toxicity
A standard MTT assay was carried out to evaluate the toxicity of modified siRNAs in HEK293 and HUVECs. The cells were plated in 96-well plates at 1×10^4 cells/well 24 hr before transfection using

Lipofectamine 2000. Twenty-four hours after transfection (containing 100 nM siRNA), MTT assays were performed as in our previous study.[35] MTT solution (2 μL, 5 mg/mL) was added into each well, and the treated cells were cultured for 4 hr. Then, 50 μL DMSO was added into each well to dissolve the formazan crystals and further incubated for 30 min. Finally, the absorbance of each well at 540 nm was measured using Infinite M200 (TECAN) with a reference wavelength of 650 nm and absolute absorbance (OD$_{net540}$) of OD$_{540}$ minus OD$_{650}$. Cell viability was calculated as

$$\text{Cell viability}(\%) = \left[OD_{net540(sample)}/OD_{net540(mock)}\right] \times 100,$$

where OD$_{net540 (sample)}$ is the absolute absorbance at 540 nm of the transfected cells, and OD$_{net540 (mock)}$ is the absolute absorbance at 540 nm of the mock control (non-transfected cells).

Fluorescence-Activated Cell Sorting
The cells were plated in six-well plate at 4×10^5 per well 24 h before transfection using Lipofectamine 2000. Four hours after transfection, the cells were washed with fresh PBS. Then cells were suspended in 800 μL PBS and transferred into a FACS Calibur flow cytometer (Becton Dickinson).

Statistical Analysis
We used GraphPad Prism 5 for statistical analysis. All data are shown as mean ± SEM. Statistical analysis was performed with Student's t test to evaluate single-factor differences between two sets of data or with one-way ANOVA followed by the Tukey post hoc test for multiple comparisons. A p value < 0.05 was considered to indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.0001).

SUPPLEMENTAL INFORMATION
Supplemental Information includes two figures and one table and can be found with this article online at https://doi.org/10.1016/j.omtn.2017.10.003.

AUTHOR CONTRIBUTIONS
H.C., Z.Y., and X.S. designed the experiments and wrote the paper. X.S. and X.W. conducted the experiments. Z.L. and Y.M. provided technical support for chemical modification.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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