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Effects of Conformational Alteration Induced by D-/L-Isonucleoside Incorporation in siRNA on Their Stability in Serum and Silencing Activity

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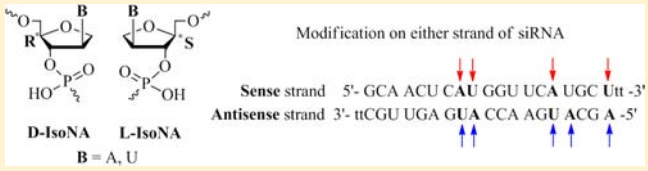
Supporting Information

ABSTRACT: We report here that all of the D- or L-isonucleoside (isoNA) modified siRNAs investigated showed the characteristic A-form conformation in the circular dichroism (CD) spectra compared to native siRNA. The D-isoNA modification had less influence on the thermal stability of siRNAs, but all L-isoNA modification displayed a significant tendency to decrease the thermal stability of siRNA. It was also found that the stabilities of D-/L-isoNA modified siMek1 in serum were different and D-isoNA modification was more potent, i.e., increase of serum stability of siRNA, than L-isoNA modification. When D-isoNA incorporated at position 4 and position 5 at antisense strand of siMek1 showed obvious improvement on serum stability, however, L-isoNA incorporated at positions 11 and 12 at antisense strand and position 9 at sense strand made the siMek1 duplex formed very unstable in serum. The silencing activities of modified siMek1s with D-/L-isoNA at position 1 of antisense strand also dropped dramatically; however, the modification at 3′-terminal of the sense strand with D- or L-isoNA significantly enhanced the silencing activity targeting the antisense strand as reporter and minimized the passenger strand-specific off-target effect. IsoNA modified in the seed area of siMek1, **siMek1 A04D** and **siMek1 A05L**, showed similar activity to the native one and better target selectivity. In the case of modification at the position near the cleavage area, it was found that D- or L-isoNA modified sense strand at position 8, 9, or 15 of siMek1 could retain the silencing activities targeting the antisense strand as reporter. Especially, both **siMek1 S15D** and **siMek1 S15L** showed good silencing activity and high target selectivity compared to native siMek1. The effects of conformational alteration of such isoNA modification of siRNA on their stability in serum and silencing activity are discussed based on computer simulation. Systematic investigation of the relationship between modified siRNA conformation and their physical and biological properties should provide a useful guideline for chemical modification and optimization of siRNA for further clinical application.



INTRODUCTION

RNA interference (RNAi) was first discovered in *Caenorhabditis elegans* where the endogenous double-stranded RNA (dsRNA) induced the sequence cleavage of complementary mRNA,[1] and later the exogenous synthetic short RNA duplex (siRNA) also showed their ability to trigger the specific target mRNA cleavage in mammalian cells.[2] The elucidation of the RNAi pathway in cells has been widely explored including phosphorylation at the 5′end of siRNA duplex by Clp1 kinase in cell cytoplasm[3−5] and then recognition of the siRNA duplex by Dicer 2 and a dsRBP-binding partner (TRBP or PACT), which cooperatively orient the siRNA duplex for strand-selective RISC loading.[6] It was proven that Dicer associated with either TRBP or PACT could sense the thermodynamic asymmetry of the siRNA duplex in which the less stable end of the duplex is preferentially bound by Dicer, while the more stable end is bound by TRBP or PACT. Next, the siRNA in Dicer/TRBP heterodimer is transferred to Ago 2 directionally with the more stable end being first transferred to a hydrophobic pocket at the Ago 2 PAZ domain from TRBP or PACT, followed by the unstable 5′ end being transferred to a conserved basic pocket at Ago 2 Mid domain. Consequently, the strand with unstable 5′ end is selected as antisense strand which has more Ago 2 protein contacts, whereas the sense strand was likely to make fewer protein contacts and thus is primed for cleavage or dissociation by the PIWI domain of Argonaute 2.

During the RISC loading and targeting process, there are specific conformational requirements for both proteins and RNAs in order to ensure proper interactions with each other. When the siRNA duplex is transferred into Ago 2 protein from Dicer/TRBP, an "open" conformation of Ago 2 protein is a

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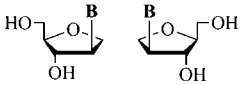
prerequisite.[7] Overall, an A-form conformation of the hybridization duplex between antisense strand and target mRNA is necessary for increased binding stability with Ago 2 protein.[8,9] It has been proven that those modification strategies which inhibited the A-form conformation of the siRNA duplex showed impaired RNAi effect.[10−13]

Although many chemical modifications on siRNA have been reported to explore the conformational requirements for protein and RNA components involved in the RNAi process,[14−17] the systematic relationship between the conformational alteration around every single nucleotide of siRNA and the biological potency has rarely been reported. Besides, the conformational alteration would also have an influence on the hybridization properties of the siRNA duplex, as well as its recognition by other proteins, such as the kinds of nucleases and RNA binding proteins. Isonucleosides (IsoNA) are a novel kind of nucleoside analogue in which the nucleobase is linked to another position of ribose other than C-1′.[18,19] In our previous work, we showed that the modified oligonucleotides D-/L-isoNA **1** and **2** (Figure 1) could form a stable duplex with DNA or RNA with a characteristic tertiary conformation as the normal unmodified DNA/RNA duplex.[20−25]



D-IsoNA **L-IsoNA**

B=Adenine (A); Uridine (U)

Figure 1. Structures of D-IsoNA and L-IsoNA.

The oligonucleotides with L-isoNA incorporated at the 3′-terminal show an increased resistance to exonuclease due to enhanced conformational stability blocking the recognition with exonuclease, whereas L-isoNA incorporated in the middle region of oligonucleotides conferred an unstable conformation of the duplex formed, which enhanced the degradation of the complementary RNA by accelerating the recruitment of corresponding RNase H.[22−24] It was also found that L-aminoisonucleoside at the 3′ or 5′ terminal of the sense strand has less effect on the RNA duplex thermal and serum stabilities, and their silencing activities are also comparable to their native siRNAs. In contrast, antisense strand modifications with L-aminoisonucleoside at the corresponding positions brought about a striking negative effect on RNA duplex stability and maintained around 40−50% of gene knockdown activities.[25] Further, a 5′-CH_2-extended chain L-IsoNA modified siRNA passenger strand at the 3′ or 5′ terminal retained the silencing activity and minimized the passenger strand-specific off-target effect.[24] Thus, in light of our previous work and other studies, it would be interesting to investigate the effects of conformational alteration caused by D-/L-isoNA modification at different positions of siRNA on their stability in serum and RNAi activity. Our planned investigation would explore the influence of conformational alternation on siRNA's properties more comprehensively and with greater accuracy.

The ERK signaling pathway proved crucial for the replication process of influenza virus, coronavirus, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, HSV-2, and so on.[26,27] For further analysis of the effects on RNAi, the sequence targeting the MEK1 kinase mRNA was studied. RNase A degradation was shown as a major degradation mechanism of siRNAs,[28] and A-U site was identified as the nuclease sensitive position of siRNA in serum, and hence minimal modification around the A-U site was expected to increase the serum stability of siRNA.[29] In order to increase the

Table 1. Sequences of D-/L-isoNA Modified siRNA[a]

	No.	Name	siRNA
sense strand (SS)	1	siMek1	SS: 5'-GCA ACU CAU GGU UCA UGC Udtdt-3'
antisense strand (AS)			AS: 5'-AGC AUG AAC CAU GAG UUG Cdtdt-3'
Sense strand modification (matched with siMek1-AS)	2	siMek1-S08D	5'-GCA ACU C^{DI}*A*U GGU UCA UGC Udtdt-3'/As
	3	siMek1-S08L	5'-GCA ACU C^{LI}*A*U GGU UCA UGC Udtdt-3'/As
	4	siMek1-S09D	5'-GCA ACU CA^{DI}*U*GGU UCA UGC Udtdt-3'/As
	5	siMek1-S09L	5'-GCA ACU CA^{LI}*U* GGU UCA UGC Udtdt-3'/As
	6	siMek1-S15D	5'-GCA ACU CAU GGU UC^{DI}*A* UGC Udtdt-3'/As
	7	siMek1-S15L	5'-GCA ACU CAU GGU UC^{LI}*A*UGC Udtdt-3'/As
	8	siMek1-S19D	5'-GCA ACU CAU GGU UCA UGC ^{DI}*U*dtdt-3'/As
	9	siMek1-S19L	5'-GCA ACU CAU GGU UCA UGC ^{LI}*U*dtdt-3'/As
Antisense strand modification (matched with siMek1-SS)	10	siMek1-A01D	SS/ 5'-^{DI}*A*GC AUG AAC CAU GAG UUG Cdtdt-3'
	11	siMek1-A01L	SS/ 5'-^{LI}*A*GC AUG AAC CAU GAG UUG Cdtdt-3'
	12	siMek1-A04D	SS/5'-AGC ^{DI}*A*UG AAC CAU GAG UUG Cdtdt-3'
	13	siMek1-A04L	SS/5'-AGC^{LI}*A*UG AAC CAU GAG UUG Cdtdt-3'
	14	siMek1-A05D	SS/5'-AGC A^{DI}*U*G AAC CAU GAG UUG Cdtdt-3'
	15	siMek1-A05L	SS/5'-AGC A^{LI}*U*G AAC CAU GAG UUG Cdtdt-3'
	16	siMek1-A11D	SS/5'-AGC AUG AAC C^{DI}*A*U GAG UUG Cdtdt-3'
	17	siMek1-A11L	SS/5'-AGC AUG AAC C^{LI}*A*U GAG UUG Cdtdt-3'
	18	siMek1-A12D	SS/5'-AGC AUG AAC CA^{DI}*U* GAG UUG Cdtdt-3'
	19	siMek1-A12L	SS/5'-AGC AUG AAC CA^{LI}*U*GAG UUG Cdtdt-3'

[a]Sites of D-/L-isoNA substitutions are indicated in *red italic*. DI represents D-form isoNA; LI represents L-form isoNA.

nuclease resistance, the vulnerable positions of siRNA were modified selectively, and to monitor the structural requirements of siRNA for silencing effects, the terminal positions, positions 4 and 5 at the center of seed region, and positions 11 and 12 around the cleavage site at the antisense strand or the sense strand of siMek1 were designed (Table 1).[30−34] To evaluate the silencing activity and passenger strand-specific off-target effect, we constructed two systems. Each system contained one strand of siRNA via siQuant vector as reporter, matched with the siRNA sense strand or antisense strand. The IsoNA modification on each strand of siRNA was carried out and tested by these two systems respectively.

MATERIALS AND METHODS

Solid-Phase Synthesis of RNA Oligonucleotides. RNA oligonucleotide synthesis was carried out on the 1 μmol scale using Applied Biosystems model 394 DNA Synthesizer according to regular phosphoramidite chemistry. The 2′-OTBDMS protected D- and L-isoNA phosphoramidite monomers were synthesized as previously reported,[20,24] then the D-/L-isoNA phosphoramidite monomers were inserted singly into the siRNA sequence at the modified position targeting to MEK1 (Table 1). Furthermore, an extended coupling time of 900 s was used for D-/L-isoNA phosphoramidites instead of the standard coupling time of 600 s used for the four standard 2′-OTBDMS protected RNA phosphoramidites (A^{Bz}, C^{Ac}, G^{Ac}, and U) due to the steric effect of isoNA phosphoramidites. Cleavage and deprotection of the oligomers were performed in concentrated ammonia at 55 °C for 24 h and TBDMS group was removed by treatment with Bu_4NF. The crude product was purified by anion-exchange high-performance liquid chromatography (Dionex, DNAPac, PA200, 9 × 250 mm) using a linear gradient of 12.5−40% eluent A in 40 min. Solutions of 0.02 M Tris-$HClO_4$ in 10% CH_3CN, pH = 8, were used as eluent B, and 0.4 M $NaClO_4$ in eluent B was used as eluent A. Then, the purified oligonucleotides were desalted by Sephadex G25 column. The purity and identity of the oligonucleotides was confirmed by ion exchange chromatography and MOLDI-TOF MS, respectively, and all single strands were HPLC purified to >90% purity. Finally, the pure oligonucleotides were lyophilized and stored at −40 °C.

Formation of siRNA Duplex and Thermal Denaturation Studies. Solutions of isoNA incorporated oligonucleotides and their complementary oligonucleotides were separately prepared in deionized water and mixed at 50 pmol/μL of the same amount. The mixture was heated to 95 °C and then cooled to 4 °C slowly to finish the annealing process. The hyperchromicity curves of the hybridized duplex solution [1 μM in T_m buffer (5.7 mM Tris-HCl, pH 7.5, 5.7 mM KCl, 0.1 mM $MgCl_2$)] were recorded on a Varian Cary 300 Bio UV−visible spectrophotometer. The absorbance was recorded in the reverse and forward directions for a temperature range of 90−25 °C at a rate of 0.3 °C/min at the settled wavelength of 260 nm.

Circular Dichroism Spectra Studies. The annealed duplexes used for circular dichroism (CD) measurements were the same as those used for the thermal denaturation studies. The circular dichroism (CD) spectra were scanned on a JASCO J-810 spectropolarimeter at 20 °C, in the 200−400 nm range.

Molecular Modeling Studies. All molecular simulations were performed on a workstation. The structures of DNA/RNA duplexes were generated after applying the AMBER 99 force field. The molecular mechanical parameters of isoNAs were obtained using *Gaussian 98* program under HF/6-31G** level of theory. Molecular dynamics (MD) simulations were carried out by using the SANDER module of AMBER 8 with SHAKE algorithm applied to hydrogen atoms. Each simulation began with 1000 steps of minimization followed by 25 ps of equilibration dynamics at 300 K with full constraints on duplex. The final structure of each duplex was obtained from the averaged structure of the last 800 ps of molecular dynamics calculation followed by 1000 steps of minimization. All the simulated duplexes were analyzed with *CURVES 5.3* program.

Serum Degradation Assay. Serum degradation assays were performed by incubating 1 μL of 20 μM siRNA in PBS buffer containing 50% FBS at 37 °C. Aliquots of 10 μL were collected after being treated for 0, 3, 6, 12, and 24 h, then the solution was immediately frozen in liquid nitrogen and stored at −80 °C until analysis. The control assay was performed for each siRNA without FBS as well. Together with 3 mL of 6× RNA loading buffer, all the samples were resolved in 20% polyacrylamide gels and visualized by SYBR.

Cell Culture. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 2% or 10% heat-inactivated fetal bovine serum (FBS, Hyclone) at 37 °C in a 5% CO_2 humidified incubator.

Transfection and Dual-Luciferase Assay. Human embryonic kidney cells (HEK293) were grown in DMEM (Life Technologies, Gibco) and seeded in 24-well plates. After the cells reached about 50% confluence, the culture medium was changed to OPTIMEM (Gibco) and then transfected with plasmids and siRNA duplex in the presence of 0.17% Lipofectamine 2000 (Invitrogen). For each well, 0.17 g of recombination plasmid and 0.017 g pRL-TK were used. The final concentration of siRNA was 10 nM. The transfection medium was changed to culture medium (1 mL) after 4 h. All experiments were tested three times and each test was repeated at least twice. Cells were harvested 24 h after transfection and lyzed with passive cell lysis. Dual-luciferase assay was conducted. Luciferase activities were determined with 10 μL cell lysate using the Dual-Luciferase Assay System (Promega) by NOVOStar (BMG Lab technologies Gmbh, Germany). The ratio between firefly and Renilla luciferase readings was generated for each well, and the inhibition efficiency of each siRNA was calculated by normalizing to respective buffer control.

RESULTS

Hybridization Properties of D-/L-isoNA Modified siRNAs. The thermal stabilities of the modified RNA duplexes prepared are listed in Table 2. The results show on comparison that D-isoNA modification has less influence on the thermal stability (T_m) of siRNAs, but all L-isoNA modifications display a significant tendency to decrease the thermal stability (i.e., $-\Delta T_m$) of siRNA.

However, siRNAs formed by the D-isoNA modified sense strand at position 19 (Table 2, **siMek1-S19D**) or L-isoNA modified antisense strand at position 1 (Table 2, **siMek1-A01L**) showed an increased T_m value of about 1.5 °C, and the duplex formed by L-isoNA at position 19 of sense strand (Table 2, **siMek1-S19L**) or D-isoNA at position 1 of antisense strand (Table 2, **siMek1-A01D**) both showed T_m values closer to that of the native siRNA duplex. It was also found that siRNA formed by two modified strands, D- or L-IsoNA modified of

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Table 2. Thermal Denaturation Studies of D-/L-isoNA Modified siRNA[a]

Name	T_m(°C)	ΔT_m(°C)	Name	T_m(°C)	ΔT_m(°C)
siMek1	59.2	-	siMek1	59.2	-
siMek1-S08D	59.2	0	siMek1-S08L	54.4	−4.8
siMek1-S09D	58.6	−0.6	siMek1-S09L	56.0	−3.2
siMek1-S15D	59.3	+0.1	siMek1-S15L	54.0	−5.2
siMek1-S19D	60.7	+1.5	siMek1-S19L	58.6	−0.6
siMek1-A01D	59.9	+0.7	siMek1-A01L	60.8	+1.6
siMek1-A04D	59.5	+0.3	siMek1-A04L	54.3	−4.9
siMek1-A05D	58.7	−0.5	siMek1-A05L	56.9	−2.3
siMek1-A11D	59.7	+0.5	siMek1-A11L	55.5	−3.7
siMek1-A12D	58.8	−0.4	siMek1-A12L	55.6	−3.6
siMek1-S09D/A11L	55.6	−3.6	siMek1-S09L/A11L	56.6	−2.6
siMek1-S09D/A11D	58.4	−0.8	siMek1-S09L/A11D	56.1	−3.1
siMek1-S19D/A01L	61.2	+2.0	siMek1-S19L/A01L	59.3	+0.1
siMek1-S19D/A01D	60.8	+1.6	siMek1-S19L/A01D	59.1	−0.1

[a]The siMek1-SD (L)/AD (L) were formed by both strands modified with isoNAs respectively, in which S and A denote the modified sense strand and antisense strands, D and L represent D-isoNA and L-isoNA, and the number shown is the isoNA incorporated position in the RNA sequence. T_m values were measured in a solution of 5.7 mM Tris-HCl, pH 7.5, 5.7 mM KCl, 0.1 mM $MgCl_2$.

both sense and antisense strands at the terminal positions (**siMek1-S19D/A01L; siMek1-S19D/A01D; siMek1-S19L/A01L; siMek1-S19L/A01D**) could conserve or increase the thermal stability compared to the native variety. All of the D- or L-isoNA modified siRNAs showed the typical A-form conformation in the circular dichroism (CD) spectrum compared to native siMek1 (Supporting Information **1**). These results show that the modification at terminal position by D- or L-isoNA is more deterministic of duplex hybridization. The local conformational alteration caused by D- or L-isoNA modification at terminal position may also change the orientation of 3′-overhang of siRNA and bear some influence on the interaction to RISC and Ago protein.

Stability of Isonucleoside (isoNA) Modified siRNAs in Serum. Many chemically modified siRNAs have shown the ability to increase the biological stability of siRNA.[35−37] It has been shown that modification strategies around the A-U site of siRNA improved the biological stability either through restricting the conditions required for the corresponding nuclease activity[38,39] or sterically blocking the interaction between the nuclease sensitive sites of the siRNA and the nuclease protein's active center.[40,41]

In our case, it was found that although all the positions modified by D- or L-isoNA were designed to be located at A-U site, the effects of D- or L-isoNA modified siRNA on serum stability were different and D-isoNA modification exhibited greater potency than L-isoNA modification, i.e., for the increase of stability in serum (Figure 2). Although D-isoNA incorporated at position 4 and position 5 of antisense strand showed obvious improvement on serum stability (Figure 2, **siMek1-A04D, siMek1-A05D**), however, L-isoNA incorporated at positions 11 and 12 of antisense strand and position 9 of sense strand made the siRNA formed very unstable in serum (Figure.2, **siMek1-A11L, siMek1-A12L, siMek1-S09L**). D-isoNA located at position 11 and position 12 of antisense strand showed no such obvious effects on their siRNA serum stability. Interestingly, both D- and L-isoNA modified at terminal position of sense or antisense strand could increase the serum stability of the siRNA formed (Figure.2, **siMek1-S19D, siMek1-S19L, siMek1-A01D, siMek1-A01L**). Positions 8 and 9 in sense strand and positions 12 and 11 in antisense strand

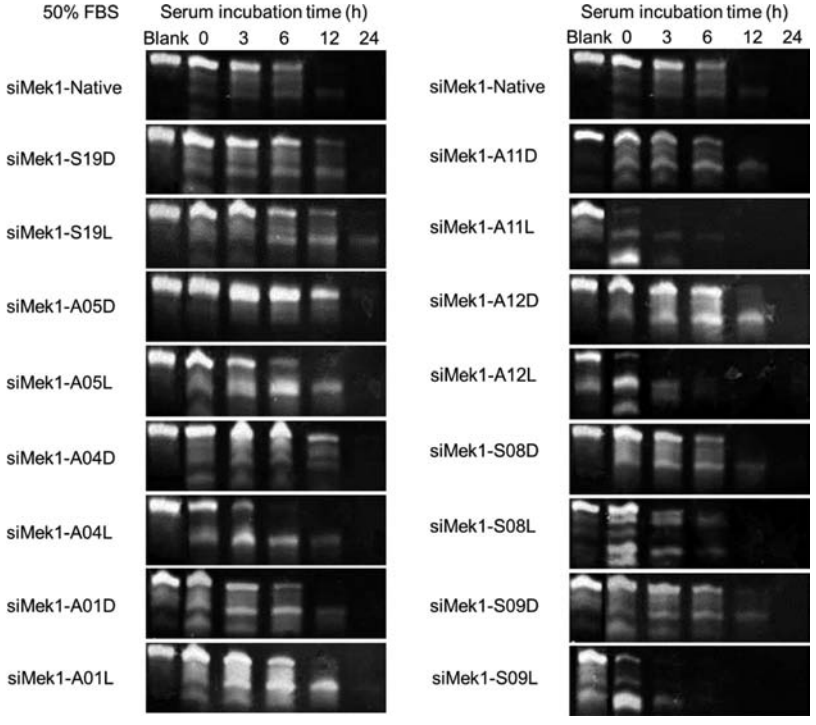

Figure 2. Serum stability of D-/L-isoNA modified siRNAs. All these siRNAs were incubated in 50% fetal bovine serum at 37 °C and withdrawn at indicated time points. The samples were resolved by PAGE and visualized with SYBR gold.

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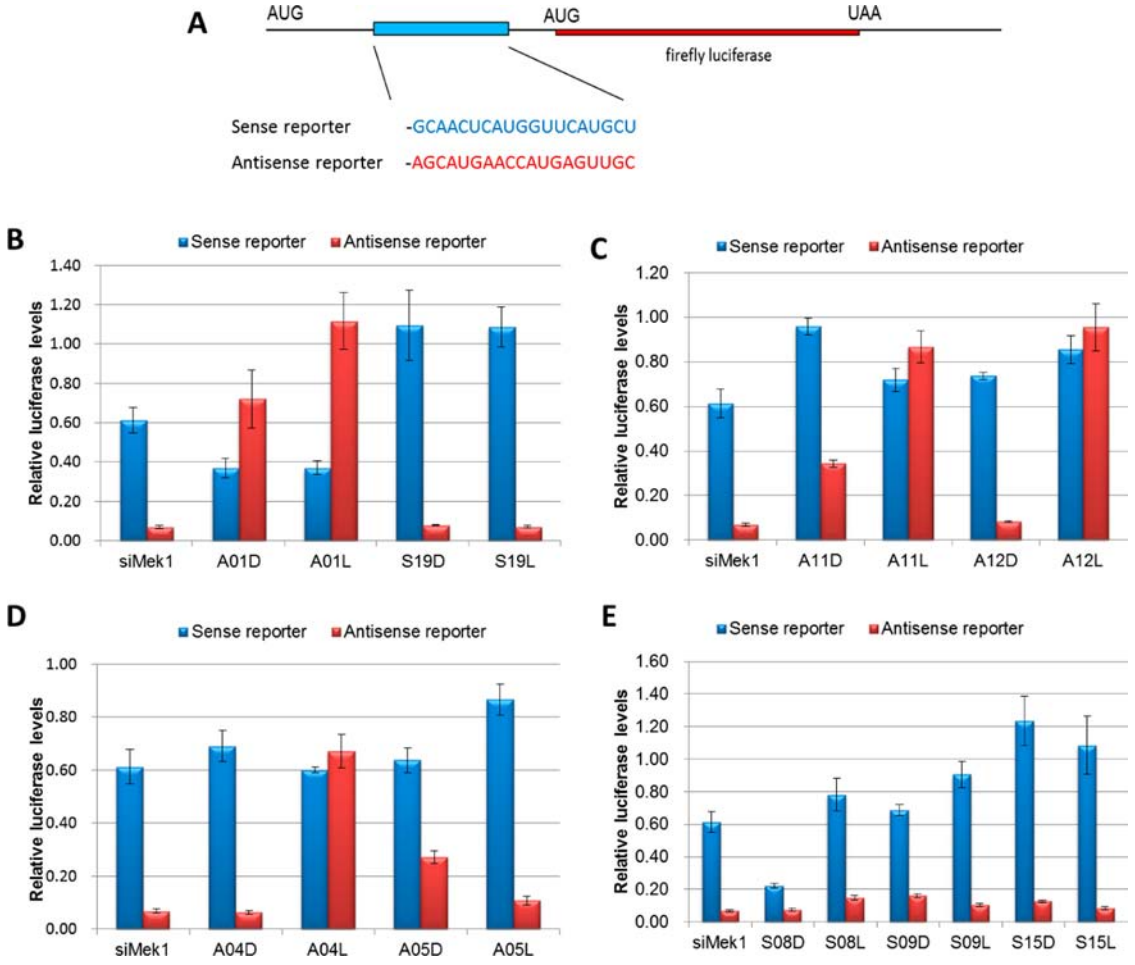

Figure 3. (A) Schematic diagram of the fusion firefly luciferase reporter used. The target sequences complementary to sense strand and antisense strand were separately fused into the plasmid. Shown to the left is the inserted in-frame AUG start codon, followed by a box representing the siRNA target site, and then as a thick line, the luciferase gene with authentic AUG codon. In (B–E), the silencing efficiencies of D-/L-isoNA modified siRNA were plotted in groups divided according to modification sites. The relative luciferase levels shown on the *Y*-axis were expressed in normalized ratios between the Renilla luciferase and the firefly luciferase activities, and they were inversely proportional to the silencing activity of siRNA. Results were average values of assays in triplicate and all the experiments were repeated three times. The blue bars represented the activities targeting sense strand as reporter tested by siMek1 and isoNA modified siMek1; the red bars represented the activities targeting antisense strand as reporter tested by siMek1 and isoNA modified siMek1.

are complementary to each other, and hence isoNA modifications at these sites showed the same effects on serum stability. These results demonstrated that the effects on serum stability of siRNA depended on isoNA modification made at specific positions along the sequence. In our case, both the terminal modifications at positions 1 and 19, either with D- or L-isoNA, provided enhanced stability over native siRNA; and D-isoNA modified at position 5 of antisense strand, **siMek1-A05D**, could form a much more stable siRNA in serum for 12 h.

Silencing Activity. siQuant assay, in which the target fragments of siRNA's sense strand and antisense strand in-frame are separately fused with the firefly luciferase gene in a mammalian expression vector (Figure 3A), was used to evaluate the silencing activity and the passenger strand-specific off-target effect.[42] The relative luciferase levels shown on the *Y*-axis (Figure 3) were inversely proportional to the silencing activity of siRNA. All the modified sites were chosen according to the predetermined sites crucial for exerting siRNA activity, such as positions 4 and 5 at the center of seed region, the 5′-terminal, positions 11 and 12 around the cleavage site at the antisense strand, together with the cleavage sites and the 3′-terminal in the sense strand.[30−34] The results in Figure 3 after the tests with D- or L-isoNA modified siMek1 at different sites reflected the silencing activities and the difference between two activities targeting sense strand reporter and antisense strand reporter reflected the passenger strand-specific off-target effect.

It was found that when the 5′-terminal of antisense strand was modified with D- or L-isoNA, the residual activities of **siMek1-A01D** and **siMek1-A01L** (Figure 3B) targeting antisense strand as reporter showed an increase from 6.78% to 71.99% and 111.57%, respectively, compared to the native one indicating the significant negative effect of isoNA modified antisense strand for silencing activity, and the residual activities of the same samples targeting sense strand as reporter were reduced from 61.26% to 36.86% for both D- and L-isoNA modification showing a slight increase of silencing activity. Interestingly, the modification at the 3′-terminal of the sense strand with D- or L-isoNA, **siMek1 S19D** and **siMek1 S19L** (Figure.3B), showed the same results targeting the antisense strand as reporter as those of the native one, but they both dramatically enhanced the silencing activity targeting the

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antisense strand reporter indicating the significant selectivity to two different targets. This shows that modification at the 3′ terminal sense strand of siMek1 could minimize the passenger strand-specific off-target effect. The results in Figure 3C showed the activities of D-/L-isoNA modified siMek1 at the adjacent positions 11 and 12 targeting the two reporters. Both L-isoNA modified antisense strands at position 11 and position 12 of siMek1, **siMek1 A11L** and **siMek1 A12L** (Figure 3C), eliminated the silencing activity irrespective of the sense strand being a reporter target or antisense strand as reporter. It seems that the effects induced by D-isoNA incorporation were less than those of its L-analogue, and the silencing activity depended on the positions modified by D-isoNA: **siMek A12D** (Figure 3C) retained the same silencing activity as the native one and **siMek A11D** showed reduced silencing activity. Position 4 and position 5 in the middle of the seed region are the important sites for siRNA's activity, and it has been proven that these positions are not well tolerated after chemical modification.[13,30] In our case, Figure.3D, **siMek1 A04L** showed no silencing activity targeting both sense strand as reporter and antisense strand as reporter; **siMek1 A05D** indicated some reduced silencing activity targeting antisense strand as reporter. However, isoNA modified siMek1, **siMek1 A04D** and **siMek1 A05L**, showed activities which are similar to the native one no matter the targeting to sense strand as reporter or antisense strand as reporter and showed better target selectivity. In the case of modification of position near the cleavage area, it was found that D- or L-isoNA modified sense strand at position 8, 9, or 15 of siMek1, **siMek1 S08D**, **siMek1 S08D**, **siMek1 S09D**, **siMek1 S09D**, and **siMek1 S015D**, **siMek1 S15L** (Figure 3E), could retain the silencing activities targeting the antisense strand as reporter. Especially **siMek1 S015D** and **siMek1 S15L** showed both good silencing activities and high target selectivity compared to native siMek1.

DISCUSSION

The process of siRNA-directed target cleavage requires the participation of many other cellular RNA strands and proteins, and the interaction between siRNA duplex and these components basically determines the biological properties such as serum stability,[28] immunogenicity,[43] and silencing activity[3,6,7,9,30−32] of siRNA. The chemical modification approaches were undertaken to optimize siRNA properties in order to probe the interaction between siRNAs and these cellular components.[9,14,28] Therefore, a thorough understanding of the relationship between conformational alteration of siRNA and its physical and biological potency is very necessary. Some of the chemical modifications at specific positions of siRNA could introduce alteration of the local conformation,[14−17] but also would bring other effects such as the alteration of electronegativity and hydrophobic property at the modified position. Isonucleosides are the stereoisomers of the native 2′-deoxynucleosides; D-isoNA and L-isoNA modified oligonucleotides show clearly the conformational change of the nucleic acid backbone without the significant influence on electronegativity and hydrophobic property. Both D-isoNA and L-isoNA modified siMek1s have shown the typical A-form conformation based on the results of CD spectrum. However, according to the results of thermal denaturation study, D-isoNA and L-isoNA modified strands indicate a different influence on the formation and thermal stability of the hybridized RNA duplex. All the hybridized RNA duplexes with single L-isoNA incorporation showed less thermal stability compared with native duplexes and the D-isoNA incorporated strands indicated a tendency to increase the thermal stability of hybridized RNA helix. The results of computer simulation also showed that D- or L-isoNA modified siMek1, **siMek1-A08D**; **siMek1-A08L**; **siMek1-A15D**; **siMek1-A15L**, maintained stable A-form duplexes as the native **siMek1** during the entire course of simulation (Figure 4). These results were also in agreement

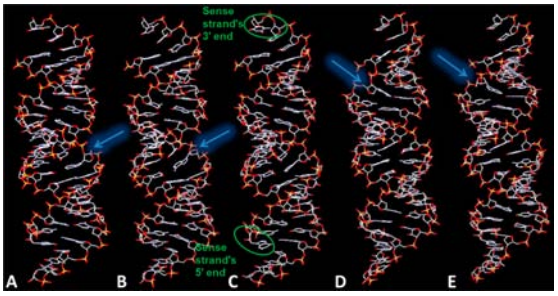

Figure 4. Predicted structures of D-/L-isoNA modified siRNAs revealed in MD simulations. The 3′ and 5′ ends of sense strand were marked with a green circle and the modified sites were pointed out with blue arrows. RMSD values indicate the structural distortion in mismatched RNA duplexes. (A) siMek1-A08D; (B) siMek1-A08L; (C) siMek1; (D) siMek1-A15D; (E) siMek1-A15L.

with the thermal denaturation and CD spectrum results. The results of computer simulation showed that the D- or L-IsoNA incorporation affected the local conformation around modified sites of siMek1 to varying degrees. Compared to native nucleoside in the RNA duplex, D-isoNA incorporation brought slightly rotational distortion of the oligonucleotide at the modified location, however, owing to the different stereo-structure of L-isoNA, the backbone of modified oligonucleotide had to rotate significantly to maintain stable base pair hydrogen bonding. The dramatic backbone distortion also affected the base stacking and backbone conformation of the adjacent nucleotides, which in turn was expected to affect the binding of the modified strand with the related proteins.

To take the dynamic simulation of the duplex with D- or L-isoNA modified at positions 8 and 9 of the sense strand separately as an example, the L-isoNA incorporation would increase the probability of mismatch pairing at the modified location and an unusual A−A, U−U base pair hydrogen bonding would be formed (Figure 5). As shown in Figure 5 (left), formation of a hydrogen bond of the adenine base of L-isoNA at position 8 of the sense strand with the adenine at position 11 of the complementary antisense strand is a 86.6% possibility in the simulation process, and formation of an uncommon U−U bonding of the uridine base of its adjacent nucleotide at position 9 with the mismatch nucleotide's base at position 12 of the complementary strand is a 85.4% possibility in the simulation process. The modification with L-isoNA at position 9 of the sense strand showed a similar result (Figure 5, right) that the uridine base at position 9 could form a mismatch U−U hydrogen bond with the uridine base at position 11 of complementary strand with 64.85% possibility in the simulation process. The probabilities of anomalous A−A and U−U base pairs occurring may provide the necessary potential energy required to maintain the distorted but stable local conformation (Supporting Information 2).

These results are overall consistent with the thermal stability data; L-isoNA incorporation in the middle of siRNA duplex indicated the reduced T_m value by 3−5 °C compared to the

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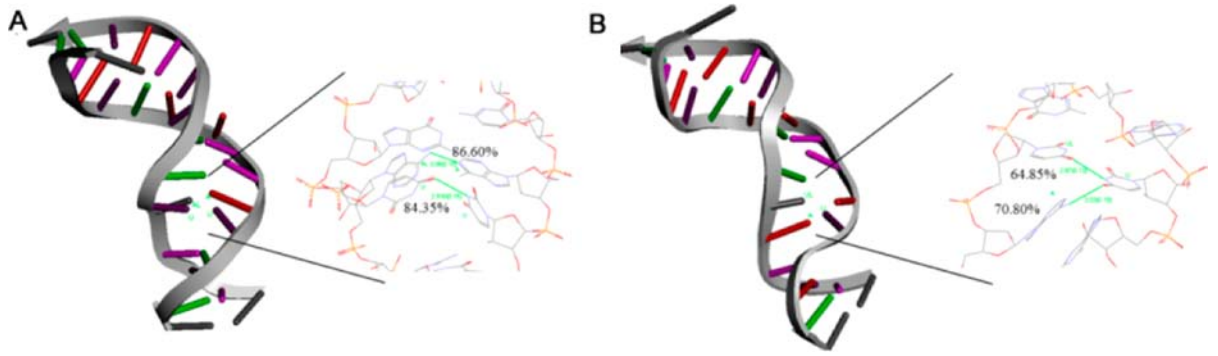

Figure 5. Probability of the mismatch base pairing due to L-isoNA incorporation explored through dynamic simulation. The percentage values near the mismatch hydrogen bond are the ratio between the sum time of establishment of the hydrogen bond and the whole time of the simulation process. (A) siMek1-S08L; (B) siMek1-S09L.

native one, while D-isoNA incorporation at the middle position of siRNA showed T_m values similar to the native duplex. The computer simulation showed that both D- and L-isoNA modified at the terminal of the sense strand or antisense strand could maintain the conformation as the native siRNA which is in agreement with the thermal stability data (Table 2). However, in active RISC complex, the 5′ nucleotide at position 1 of the antisense strand flips out of the A-form duplex and the 5′-P on the antisense strand is recognized and anchored by a nucleotide specificity pocket at Ago 2 Mid domain. The binding pocket is rather rigid in that its conformation is nearly unchanged whether there is nucleotide inside or not,[31] which means there is a strict conformation requirement for the inside 5′ nucleotide. According to the results from Figure 3B, the silencing activity of modified siMek1s with D-/L-isoNA at position 1 of the antisense strand dropped dramatically. The conformational distortion caused by 5′-isoNA modification may affect the process of 5′-terminal phosphorylation[3] or inhibit interaction of the 5′-phosphate groups with the rigid 5′-phosphate-binding pocket,[31] and both D- and L-isoNA modifications may disturb the assembly of RISC; even the conformation of D-isoNA modified strand is very similar to that of the native one. However, when D-/L-isoNA is modified at position 19 of the sense strand, an enhancement of selective silencing activity was obvious, which may indicate competition between the two strands of siRNA during the process of assembly into RISC complex,[24,37] and the conformation of D- and L-isoNA modified sense strand may be more suitable to the recognition of siRNA to RISC for strand selection.

It was reported by the studies of crystal structure that binary Ago complex with antisense strand showed that residues 2 to 10 of the antisense strand are continuously stacked but with distinct breaks at the 1−2 and 10−11 steps.[32] However, the ternary Ago complex with additional target RNA showed that Ago-mediated target RNA cleavage requires Watson−Crick pairing between guide strand and target RNA as an A-form conformation spanning both the seed segment (positions 2 to 8) and the cleavage site (positions 10 and 11).[30] Therefore, with the additional target RNA, the nonhelical conformation at position 10−11 in the binary Ago complex is released on ternary Ago complex formation to adapt into the undistorted helical conformation requirement at the cleavage site.[9] It was reported that position 4 and position 5 in the middle of the seed region are the important sites for siRNA activity, and it has been proven that these positions are not well tolerated for chemical modification.[13,30] In our case, D-isoNA modified antisense strand at 4 or 12 position also led to more silencing activity of siMek1 and eliminated the passenger specific off-target effect compared with the native one. It seems that the conformation of D-isoNA modified strands of siMek1 at these positions could be more suitable to the conformation requirements for siRNA silencing activity and L-isoNA modified strands at position 5 could also meet these requirements. According to the crystal structure study of the ternary Ago complex with additional target RNA, positions 12−17 of antisense strand were shown few intermolecular contacts with the Ago 2 protein, but in our case, Figure 3C,E showed that modification at position 12 led to more influence on the silencing activity. D- or L-isoNA modified sense strand at position 15 or 19 lent siMek1 more silencing activity and significantly minimized the passenger specific off-target effect. It was noticed that 2−4 bp on both ends of a siRNA are important for their relative loading efficiency into RISC and the thermodynamic properties of 2−4 bp on both ends of effective siRNAs also were proven as determinants for pre-RLC recognition and affect overall silencing activity.[44,45] Our observations are consistent with these reports. The difference of antisense strand silencing activities between L-isoNA modification at position 4 and position 5 may indicate that a flexible regional conformation of such a modified strand played a different role in the pre-RLC recognition.

Native siRNAs have been reported to be quite unstable in serum, which limits siRNAs for further clinical application.[38] Many chemical modification strategies have been studied in order to improve siRNA serum stability. According to the stability study in serum, D- or L-isoNA modified at the terminal end of both sense strand and antisense strand showed greater stability compared to the native one; especially, D-isoNA modified at position 5 of the antisense strand was very stable in serum for 12 h. RNase A which preferentially attacks the A−U site in single-stranded RNA was confirmed as the key nuclease responsible for cleavage of the siRNA duplex in serum.[28] It was proposed that the degradation of siRNAs by RNase A is processed when the duplex transiently "breathes" apart at the A−U site. To discover the effect of area on the conformation requirement of D-isoNA modification, the free energy profile of each site was calculated utilizing Sfold (http://sfold.wadsworth.org/cgi-bin/index.pl) (Supporting Information 3). As shown by computer simulation (Figure 5), for L-isoNA modified at positions 8 and 9 of the strand, the original compacted duplex would be affected by the flexible conformation at the modified location, which would increase the possibility for nuclease

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recognition and attack. The exploration of the conformation requirement at different areas of siMek1 in terms of thermal stability, nuclease recognition, and silencing activity would provide important clues for the improvement of an effective siRNA therapy. Interestingly, D-isoNA modified at position 8 of the sense strand of siMEK1 (**siMek1 S08D**) showed very good anti HSV activity in two cell lines (data not shown).

CONCLUSIONS

D- and L-isoNA are stereoisomers and have the same constitution as the native deoxynucleosides; all of the D- or L-isoNA modified siRNAs showed the typical A-form duplex by circular dichroism (CD) spectrum compared to native siMek1. The D- and L-isonucleoside (isoNA) modifications induced various influences on the local conformation of the siMek1 duplex, and thus affected their thermal stability, stability in serum, and silencing activity. Therefore, the properties of D-/L-isoNA modified siRNA at the different sites could provide us the requirement of regional conformation for RNAi. Systematic investigation of the relationship between modified siRNA conformation and the physical and biological properties may provide a useful guideline for chemical modification and optimization of siRNA for further clinical application.

ASSOCIATED CONTENT

Supporting Information

Circular dichroism spectra of D-/L-IsoNA modified siRNAs; predicted regional molecular structures of D-/L-isoNA modified siRNA; Sfold calculated internal stability profile. 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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