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RNA interference in mammalian cells by siRNAs modified with morpholino nucleoside analogues

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ABSTRACT

siRNAs modified with morpholino nucleoside analogues were synthesized and their biological properties were examined in details. The gene silence abilities of modified siRNAs were correlated to the positions of the modifications, some of which appeared to be more potent than the native siRNA. The 3′-end modification improved the stability of siRNAs in serum significantly. Furthermore, the dose–response and time-course experiments demonstrated that the siRNAs with 3′-end modification have potent gene silence activity at lower concentration and prolonged action time. These favorable properties make the morpholino modified siRNA a potentially useful tool in therapeutic applications.

1. Introduction

Small interfering RNAs (siRNAs) are a class of short double-stranded RNA molecules involved in the RNA interference (RNAi) pathway, which leads to the target mRNA degradation.[1–7] Although siRNAs have become powerful tools in biological research,[8–11] their applications as therapeutic agents were partially limited by their poor stability against nucleases.[12] Therefore, chemical modification of synthetic siRNAs to improve nuclease stability, potency, specificity and in vivo cellular delivery emerged as an active research area. Many chemical modifications have been reported (reviewed in Refs. 13–15), such as thiophosphate,[16] boranophosphate,[17] 4′-thio,[12,18] locked nucleic acid (LNA),[19] 2′-alkyl[20] and 2′-deoxy-2′-fluoro.[20,21]

Morpholino oligonucleotides with morpholino rings in subunits instead of the ribose have become one of the promising candidates for biological research, because they inhibit the translation of target mRNA by steric blocking and have high resistance to enzymatic degradations.[22] In our previous work, we have synthesized and investigated the properties of chimeric oligonucleotides containing morpholino thymidine analogues.[23] These modified oligonucleotides have shown moderate thermal stability with complementary RNA and DNA, as well as enhanced resistance towards nucleases. The favorable properties prompted us to further evaluate the gene silence activity of such modified siRNAs. Here we report the synthesis, bioactivity evaluation and the structure-and-activity relationship of a series of morpholino modified siRNAs.

2. Result and discussion

2.1. Chemistry

Synthesis of the building block, phosphoramidite uridine nucleoside monomer **6** is shown in Scheme 1 following our previous procedure.[23] Briefly, uridine (**1**) was first treated with DMTr–Cl in anhydrous pyridine for overnight under argon atmosphere to afford 5′-*O*-DMTr-uridine (**2**) in 83% yield, which was then subjected to the construction of the morpholino ring in one step by treating it with sodium periodate and then ammonium biborate to afford 2′,3′-dihydroxyl-morpholino-uridine (**4**). Reduction of 2′ and 3′ hydroxyl groups on compound **4** gave compound **5**.[24,25] The total yield of the above 3 steps was 78%. The building block monomer **6** was then obtained by phosphitylation of compound **5** under the conditions with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite and 4,5-dicyanoimidazole (DCI) in anhydrous CH_2Cl_2 for 4 h under argon atmosphere with a total yield of 86%. All compounds were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and HRMS.

These modified building blocks were incorporated into siRNAs in order to test their effects on gene silence and stability. RNA oligonucleotides (sequences of sense and antisense strands are shown in Scheme 2) were synthesized on a DNA synthesizer via

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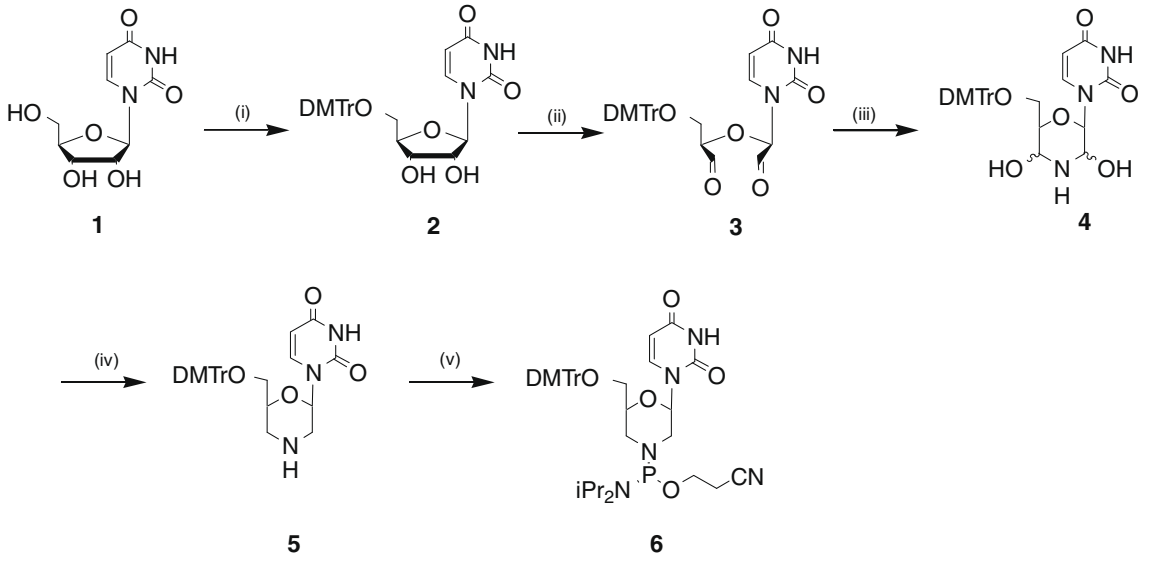


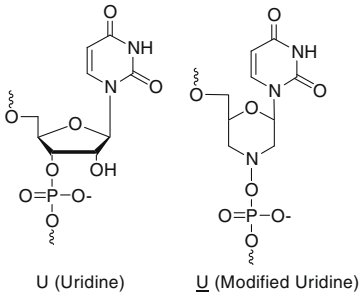
Scheme 1. Synthesis of the morpholino uridine building block. Reagents and conditions: (i) DMTr-Cl, NEt_3, Py, rt, Ar; (ii) and (iii) $NaIO_4$, $(NH_4)_2B_4O_7$, CH_3OH, rt; (iv) $NaCNBH_3$, CH_3OH, rt; (v) 2-cyanoethyl-*N,N,N′,N′*- tetraisopropylphosphorodiamidite, DCI, CH_2Cl_2, rt, Ar.

phosphoramidite route, using 5-ethylthio-1*H*-tetrazole (ETT) as the activator in 10-min coupling time. After the removal from solid support, crude oligonucleotides were purified by HPLC and analyzed by MALDI-TOF mass spectroscopy (Table S1 in Supplementary data). siRNA duplexes were obtained by annealing of equal molar of sense and antisense strands, which were then purified by HPLC.

2.2. Gene silence by modified siRNAs

To evaluate the gene silence effect of modified siRNAs, one native siRNA**7** and 13 modified siRNA**8–20** were analyzed for their ability to inhibit luciferase expression in HeLa cells (Fig. 1). Cells were co-transfected with luciferase vector and siRNA (25 nM), followed by the luciferase activity measurement after 24 h post-

Native	**7**	5' CUU ACG CUG AGU ACU UCG Att 3' 3 ' tt GAA UGC GAC UCA UGA AGC U5'
3' modification	**8**	5' CUU ACG CUG AGU ACU UCG AUU 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	9	5' CUU ACG CUG AGU ACU UCG Att 3' 3' UU GAA UGC GAC UCA UGA AGC U 5'
	10	5' CUU ACG CUG AGU ACU UCG AUU 3' 3' UU GAA UGC GAC UCA UGA AGC U 5
antisense modification	**11**	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	12	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	13	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	14	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
sense modification	**15**	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	16	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U5'
	17	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	18	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	19	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'
	20	5' CUU ACG CUG AGU ACU UCG Att 3' 3' tt GAA UGC GAC UCA UGA AGC U 5'



Scheme 2. Sequences and the chemical structure of morpholino modified siRNAs. siRNAs are shown with the sense strand above (5′–3′) and the antisense strand below (3′–5′). RNA is in uppercase letter, DNA is in lowercase letter, and modification is in uppercase letter with underline.

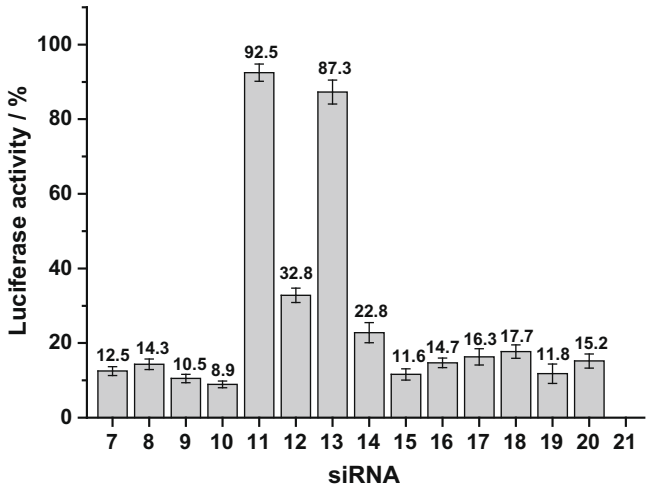


Figure 1. Effects of the morpholino modification on silencing activity. Luciferase activity was assessed according to the Dual-Luciferase Reporter Assay protocol.

transfection. The native siRNA**7** inhibited the activity of luciferase to about 12.5%, while as a comparison, some modified siRNAs appeared to have comparable or better inhibition effect and others showed decreased activity. The results suggest that the silencing activities of siRNAs correlated with the positions of the modifications as described in the following structure-and-relationships: (1) siRNAs with the sense strand modification at different positions (siRNA**15**–**20**) demonstrated good inhibition effect with the luciferase activity in the range of 11.6–17.7%, which is similar as the native siRNA**7**. In particular, siRNA**15** which has the morpholino uridine modified at the 5′ end appeared to be the most potent, whereas siRNA**17** and **18** which have the modification in the middle of the strands were a little less potent than the native siRNA**7**. This result is consistent with other reports.[19–21] (2) Modification on the 3′-overhangs in either antisense or both sense and antisense strands revealed better inhibitory activity compared to siRNA**7**. Specifically, siRNA**9** and siRNA**10** showed more potent effect than siRNA**7**. (3) Modification on the antisense strand led to a significant decrease of inhibitory activity, especially for siRNA**11** and **13**. It was reported that the cleavage site of the antisense strand is between position 10 and 11 starting from 5′-terminal.[26] siRNA**13** with modification at the cleavage site showed significant loss of gene silencing activity. Phosphorylation at antisense strand 5′ end is crucial for siRNA function.[27] Our finding showed that siRNA**11** with modification at antisense strand 5′ end significantly reduced the inhibitory activity, due to a lack of 5′ phosphorylation. Overall speaking, the sense strand was shown to have better tolerance of chemical modification, which is in agreement with other reports.[19–21]

2.3. Bio-stability in serum

Bio-stability is crucial for therapeutic application of siRNAs. The stability of modified siRNAs was evaluated using siRNA**7** as a control in serum. Based on the fact that native siRNAs degrade primarily via 3′ exonuclease in serum,[21] it is reasonable to propose that the protection at 3′ end would prevent siRNA from degradation. siRNA**9** and **10** with morpholino modification at 3′ end were chosen, because they showed improving gene inhibition effect compared to the native siRNA. As shown in Figure 2a and b, native siRNA**7** degraded rapidly with a half-life time at about 45 min, while on the contrary, both modified siRNA**9** and **10** showed significantly enhanced resistance to degradation with half lives at about 200 and 300 min, respectively (Table 1). The longer half-life times

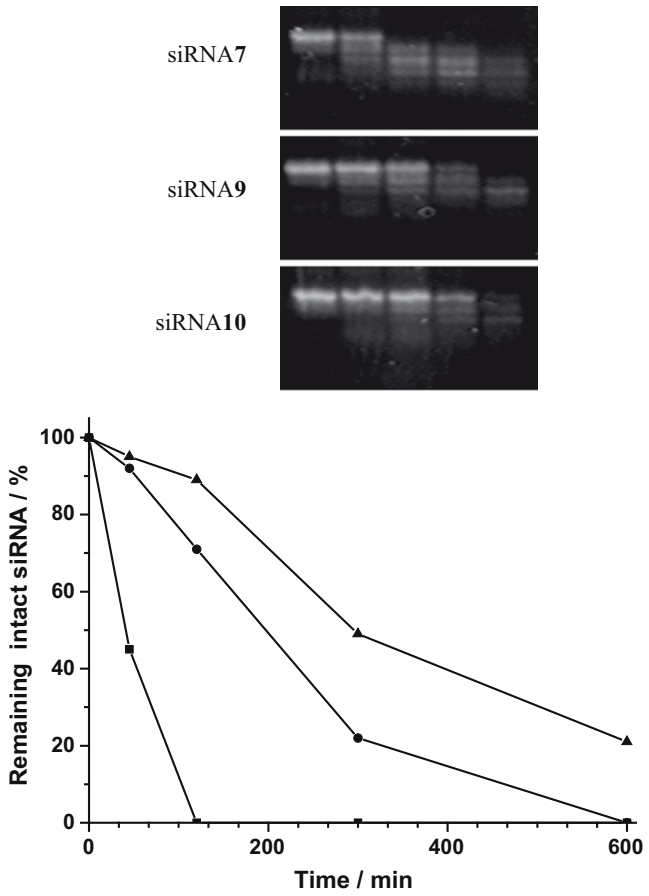


Figure 2. Stability of siRNA**7** (■), siRNA**9** (●) and siRNA**10** (▲) in 10% fetal bovine serum at 37 °C. siRNAs were taken at indicated time points, separated by PAGE and stained with GelRed.

Table 1
Half-lives[a] and IC_{50} values of siRNA**7**–**10**

Compound	Half-life (min)	IC_{50} (nM)
siRNA**7**	45	8.1
siRNA**8**	—	8.3
siRNA**9**	200	5.1
siRNA**10**	300	4.6

[a] Duplexes of siRNA (2 nmol) were incubated at 37 °C in 10% fetal bovine serum (Hyclone) diluted in phosphate buffered saline (PBS) for the corresponding time.

of siRNA**9** than siRNA**10** indicate that the 3′ end modification on both sense and antisense strand could increase the stability against exonucleases in serum.

2.4. Dose–response and time–response

Dose–response of modified siRNAs **8–10** was compared with the native siRNA**7** (Fig. 3). Generally speaking, the dose–response curves of siRNAs **8–10** are similar to that of the native siRNA**7**. All siRNAs demonstrated good inhibition effect at the concentration of 10 nM, and all siRNAs showed enhanced inhibition effect as the concentration increased and reached to the maximum activity when the concentration is about 50 nM. This result indicated that the modified siRNAs inhibited the luciferase activity through RNAi mechanism instead of other non-specific effects. Furthermore, IC_{50} values were obtained from the dose–response experi-

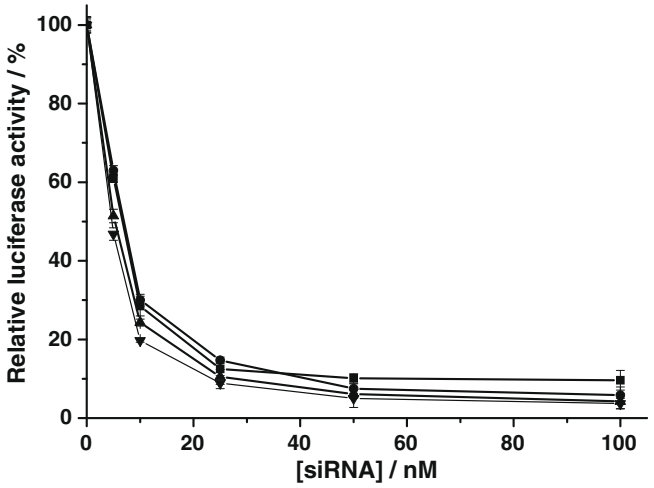


Figure 3. siRNA dose–response as measured by the decrease in luciferase activity. The native siRNA**7** (■) and morpholino modified siRNA8 (●), siRNA**9** (▲) and siRNA**10** (▼). Data was from three independent experiments.

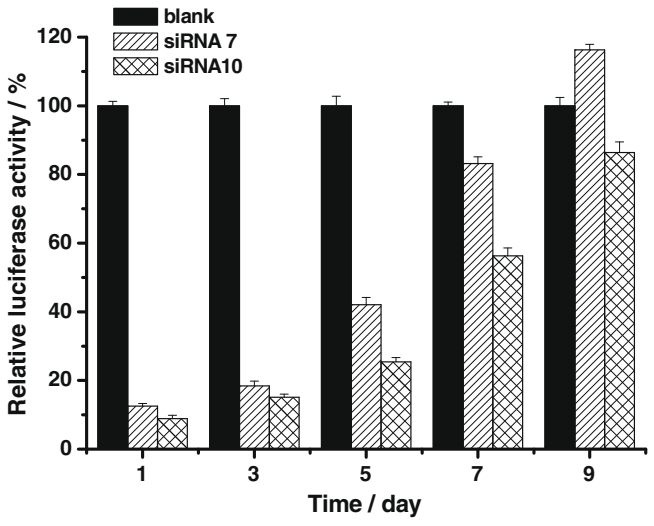


Figure 4. Time-course experiment of siRNA**7** and siRNA**10**. Luciferase activity was measured after 1, 3, 5, 7 and 9 days post-transfection. Data was from three independent experiments.

ments (Table 1). As a result, siRNA**9** and siRNA**10** have IC_{50} values at 5.1 and 4.6 nM, respectively, which are lower than that of siRNA**7** (8.1 nM).

Time-course experiments to measure luciferase activity at different days after transfection were carried out (Fig. 4). During the complete time-course, the most potent siRNA**10** showed better inhibition effect compared to the native siRNA**7**. Even at day 9 when the luciferase activity was not inhibited at all for the native siRNA**7** treated sample, the modified siRNA**10** treated sample still showed a 15% luciferase inhibition. The longer effective time of the modified siRNA might be due to its better stability.

3. Conclusions

In conclusion, we synthesized a series of siRNAs containing morpholino modified uridine analogues. It was found that the siRNA with morpholino modification at 3′ end in both sense and antisense strand appeared to be most potent and most stable against exonucleases in serum. The results suggest that morpholino modified siRNAs might find further applications in the development of synthetic siRNAs in gene therapy.

4. Experimental

4.1. Chemistry

^{1}H NMR and ^{13}C NMR were recorded in the indicated deuterated solvent at 300 MHz JEOL NMR spectrometers. Chemical shifts are expressed in ppm (d) and coupling constants *J* in Hertz (Hz). High-resolution mass spectra were obtained with Waters micromass Q-Tof Premier mass spectrometer. Reaction progress was monitored using analytical thin-layer chromatography (TLC) on pre-coated silica gel plates and the spots were detected under UV light (254 nm). All other reagents and analytical grade solvents were bought from commercial sources and used without further purification unless indicated. Methylene chloride, pyridine and triethylamine were distilled from calcium hydride (CaH_2) prior to use.

4.1.1. Synthesis of 5′-*O*-DMTr-uridine (2)

Uridine (**1**) (2.44 g, 10 mmol) was co-evaporated three times with anhydrous pyridine. Then it was dissolved in anhydrous pyridine (50 ml), and 4,4′-dimethoxytrityl chloride (5.0 g, 15 mmol), anhydrous triethylamine (2.1 ml, 15 mmol) were added. The mixture was stirred overnight at room temperature under argon atmosphere. 5% $NaHCO_3$ solution (50 ml) was added and extracted with CH_2Cl_2 (3 × 50 ml). The organic phase was washed with brine (30 ml), dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography ($CHCl_3$–CH_3OH = 15:1) to afford **2** (4.43 g, 8.1 mmol, 81%).

^{1}H NMR(300 MHz, $CDCl_3$): 3.49 (m, 2H, 5′-CH_2), 3.75 (s, 6H, OCH_3), 3.85 (s, 1H, OH), 4.17 (m, 1H, 4′-CH), 4.37 (m, 1H, 3′-CH), 4.42 (m, 1H, 2′-CH), 5.34 (d, 1H, 5=CH), 5.50 (s, 1H, –OH), 5.92 (d, 1H, 1′-CH), 6.79–6.82 (m, 4H, ArH), 7.16–7.39 (m, 9H, ArH), 7.97 (d, 1H, 6=CH), 10.5 (s, 1H, 3-NH).

^{13}C NMR (75 MHz, $CDCl_3$): 55.3 (OCH_3), 62.1 (C5′), 69.9 (C2′), 75.4 (C3′), 83.6 (C4′), 87.1 (DMTr), 90.2 (C1′), 13.4, 127.2–130.3, 135.3, 135.5, 144.5, 158.7 (DMTr), 102.4 (C5), 140.7 (C6), 151.4 (C2), 164.2 (C1).

ESI-HRMS $[M+Na]^+$ *m*/*z* Calcd for $C_{30}H_{30}N_2O_8Na$: 569.1900, Found: 569.1896.

4.1.2. Synthesis of 5′-*O*-DMTr-morpholino uridine (5)

5′-*O*-DMTr-uridine (**2**) (2.73 g, 5 mmol) was dissolved in 90 ml methanol. Sodium periodate (1.1 g, 5.1 mmol) and ammonium biborate (1.3 g, 5.7 mmol) were added with stirring. After stirring for 2.5 h at room temperature, the mixture was filtered and sodium cyanoborohydride (0.43 g, 6.8 mmol) was added to the filtrate. After 15 min, another portion of sodium cyanoborohydride (0.11 g, 1.7 mmol) was added. The reaction mixture was stirred for 6 h at room temperature, followed by evaporation. The residue was dissolved in ethyl acetate (100 ml), washed with brine (3 × 50 ml). The organic phase was dried over Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography ($CHCl_3$–CH_3OH = 20:1) to afford **5** (2.01 g, 3.8 mmol, 76% in three steps).

^{1}H NMR (300 MHz, $CDCl_3$): 2.56–2.62 (m, 2H, 2′-CH_{ax}, 3′-CH_{ax}), 3.00–3.09 (m, 2H, 3′-CH_{eq}, 5′-CH), 3.22 (m, 2H, 2′-CH_{eq}, 7′-CH), 3.78 (s, 6H, OCH_3), 3.97 (m, 1H, 4′-CH), 5.71 (d, 1H, 1′-CH), 5.75 (d, *J* = 8.25 Hz, 1H, 5=CH), 6.79-6.84 (m, 4H, ArH), 7.26–7.45 (m, 9H, ArH), 7.48 (d, *J* = 8.25 Hz, 1H, 6=CH).

^{13}C NMR (300 MHz, $CDCl_3$): 47.1 (C3′), 49.4 (C2′), 55.3 (OCH_3), 64.5 (C5′), 78.2 (C4′), 80.9 (C1′), 86.2 (DMTr), 102.5 (C5), 113.2,

127.0–130.1, 135.8, 135.9, 144.8, 158.6 (DMTr), 139.8 (C6), 150.1 (C2), 163.2 (C4).

ESI-HRMS $[M+Na]^+$ *m/z* Calcd for $C_{30}H_{31}N_3O_6Na$: 552.2111. Found: 552.2134.

4.1.3. Synthesis of 5′-O-DMTr-morpholino uridine-*N*-[(2-cyanoethyl) *N,N*-diisopropyl] phosphoramidite (6)

5′-*O*-DMTr-morpholino uridine (**5**) (1.6 g, 3 mmol) was dried in vacuum overnight. It was dissolved in anhydrous CH_2Cl_2 (30 ml) and 2-cyanoethyl-*N,N,N′,N′*-tetraisopropyl-phosphorodiamidite (1.1 ml), 4,5-dicyanoimidazole (DCI, 11 mg, 1.5 mmol) were added to the solution. The reaction was allowed to stir for 4 h under argon atmosphere, then diluted with CH_2Cl_2 (20 ml), washed with 5% $NaHCO_3$ solution (30 ml) and brine (3 × 30 ml). The organic phase was dried over Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography (*n*-hexane–ethyl acetate = 1:1, with 1% triethylamine) to afford **6** (2.0 g, 2.7 mmol, 90%).

^{31}P NMR (300 MHz, $CDCl_3$): δ = 126.1, 127.9 (two isomers, 1:1).

ESI-HRMS $[M+Na]^+$ *m/z* Calcd for $C_{30}H_{31}N_5O_7PNa$: 752.3189. Found: 752.3209; $[M+H+NEt_3]^+$ *m/z* Calcd for $C_{45}H_{64}N_6O_7P$: 831.4574. Found: 831.4571.

4.2. Synthesis, deprotection and purification of siRNA

Single strand RNAs were synthesized on ABI 3900 DNA Synthesizer at 0.1 μmol scale, using phosphoramidite approach. 0.1 M solution of phosphoramidate **6** in anhydrous acetonitrile was used for synthesis of modified oligonucleotides, and 5-ethylthio-1*H*-tetrazole (ETT) was used as an activator. When phosphoramidate **6** was incorporated into oligonucleotides, two portions of phosphoramidate solutions were delivered followed by 15 min coupling time. Oligonucleotides with modifications in the 3′-terminal ends were synthesized using universal CPG.

Oligonucleotides were removed from CPG using aqueous ammonia at 55 °C for 12 h, and the protecting groups was removed at the same time. Crude oligonucleotides were purified by HPLC (LiChrosphere 100, RP-18, 4.6 × 250 mm, flow 1 ml/min, eluent A: water, eluent B: acetonitrile, gradient started from A:B = 100:0, ended with A:B = 30:70 over 40 min). Oligonucleotides were analyzed by HPLC and mass spectroscopy.

siRNA duplexes were obtained by annealing of equimolar amounts of sense and antisense strands. The sense and antisense strands were mixed in 10 mM sodium phosphate and 100 mM NaCl buffer (pH 7.2), with a concentration of 2 μM for each strand. The mixture was heated to 90 °C for 15 min and then cooled slowly to room temperature. The annealed duplex samples were stored at 4 °C.

4.3. Biology

4.3.1. Serum stability

Duplexes of siRNA (2 nmol) were incubated at 37 °C in 10% fetal bovine serum (Hyclone) diluted in phosphate buffered saline (PBS) for the corresponding time. After incubation, samples were immediately frozen in 1.5 × TBE-loading buffer. Samples were subjected to electrophoresis in 15% polyacrylamide-TBE under non-denaturing conditions and visualized by staining with Gel Red (Biotium) and quantified by density measurement using the software of GELWORKS 4.0.

4.3.2. Cell culture and transfection

Hela cells were cultured in DMEM (Dulbecco′s modified Eagle′s medium; pH 7.2, Invitrogen) supplemented with 10% (v/v) fetal calf serum (Hyclone) and 0.5% penicillin–streptomycin. Cells were grown at 37 °C with 5% CO_2.

All transient transfections were performed using Lipofectamine 2000 (Invitrogen) with a 20:1 (pmol:μl) ratio of siRNA and Lipofectamine 2000. 2×10^5 cells per well were seeded in 12-well plates for 24 h before transfection. Each well of cells were transfected with 100 ng of pRL-SV40 (as transfection control plasmid which contains Renilla luciferase gene) and pGL3-control (as reporter vector which contains Firefly luciferase gene) and different quantity of siRNA duplexes. Twenty-four hours or longer times post-transfection, the media were aspirated and cells were washed twice with 1 × PBS. The PBS was aspirated and 250 μl of passive lysis buffer (Promega) was added and left for 15 min. Lysed cells were transferred to a 96-well assay plate for luciferase activity assay.

4.3.3. Luciferase activity assay

Luciferase activity was assessed according to the Dual-Luciferase Reporter Assay protocol (Promega) using a NovoSTAR 96-well format luminometer with substrate dispenser. A 10 μl sample were placed in each well of a 96 well plate subsequent to which 50 μl Luciferase Assay Reagent II (substrate for firefly luciferase) was added to each well by the luminometer and the firefly luciferase activity was measured. Then 50 μl Stop and Glow reagents (stop solution for firefly luciferase and substrate for Renilla luciferase) were added and Renilla luciferase activity was measured. The mean values of the luciferase activities measured for 10 s (100 reading) each were used to calculate ratios between firefly and Renilla luciferase.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.001.

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