Synthesis, physicochemical and biological properties of oligonucleotides incorporated with amino-isonucleosides

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Received August 30, 2011; accepted October 10, 2011; published online December 2, 2011

Antisense oligonucleotides (ASONs) and siRNAs have been applied extensively for the regulation of cellular and viral gene expression, and RNAi is currently one of the most promising new approaches for anti-tumor and anti-viral therapy. In order to improve bioactivity properties and physicochemical properties of siRNA, we synthesized a novel class of ASONs II–VII incorporated with amino-isonucleoside (isoA1 and isoA2) for investigation on basic physicochemical properties. Then we designed amino-isonucleoside (isoA1, isoA2 and isoT1) incorporated siRNA 2–7. Some meaningful results have been obtained from the physicochemical property experiments in ASONs. In RNAi potency experiments, we investigated RNAi potency of each strand of the siRNA. These amino-isonucleosides incorporated siRNAs showed promising bioactivity properties and had position specificity. Reduced off target effect from sense strand loading in siRNA application was observed.

1 Introduction

Oligonucleotides, such as antisense oligonucleotides (ASONs) and siRNAs have been applied extensively for the regulation of cellular and viral gene expression, and RNAi technology is becoming one of the most promising new approaches for the therapy of many irremediable diseases [1–3]. Both ASONs and siRNAs take action by hybridizing to mRNA targets by Watson-Crick base pairing and inhibit translation of mRNA in a sequence-specific manner [2, 4]. Native oligonucleotide shows low enzymatic stability and cellular permeability [5, 6]. There are many problems associated with the effective use of siRNA for in vivo application, such as off-target effects, cytotoxicity, and poor pharmacokinetic properties [7]. Chemical synthesis of siRNA using phosphoramidite building blocks to produce single-stranded oligonucleotides followed by annealing to form duplexes is a generally accepted approach in this research [8]. This approach permits incorporation of a wide variety of natural and artificial modifications into the siRNA that can help solve some of the problems associated with administration of synthetic nucleic acids into cells or animals [9].

Isonucleosides represent a novel class of carbohydrate-modified nucleoside in which the nucleobase is linked to various positions of ribose other than C-1′, and some of these nucleosides have shown interesting biological activities [10–14]. In our previous reports regarding the synthesis and incorporation of isonucleosides into antisense oligonucleotides, we found that modification of the terminal group confers strong nuclease resistance, while modification in the central region of the antisense oligonucleotide with L-isonucleoside enables it to become good substrates of RNase H [15]. Introduction of an amino group into the isonucleoside may form a Zwitterionic molecule, and increase the thermal stability of the isonucleoside-modified oligonucleotide with its complementary sequence. This is because in the physio-
logical conditions, the positively charged amino moiety should interact with the negatively charged phosphate backbone more strongly than the uncharged hydroxyl group [16]. To be used as a probe, the amino group can also be attached with a fluorescent group such as pyrene to monitor RNA folding [17]. We had reported the amino-isonucleoside isoA₁ (Figure 1) incorporated siRNA (related to 3′ end of the coding sequence of the wee1 mRNA), and found that amino-isonucleoside modification at the 3′ or 5′ terminal of sense strand showed less effects on RNA duplex thermal and serum stabilities, and their functional activities are also comparable to their native siRNAs. However, the modification on the antisense strand at the corresponding positions showed a dramatic negative RNAi potency [18].

Here, we investigate the physicochemical and biological properties of the amino-isonucleoside (isoA₁ and isoA₂) modified oligonucleotides and add another kind of amino-isonucleoside structure which has an elongated amino side chain to assist the analysis of influence of amino-isonucleoside modification on the oligonucleotides. Upon incorporating more than one isonucleosides on the same oligonucleotides strand, the formation of duplex would be blocked by the tortuosity of L-isonucleoside. For this reason, we incorporate only one amino-isonucleoside at a time in this study. Modifications of the sense strand were reported to be tolerated and the cleavage site (10–11th) and seed region (2nd–8th) of antisense strand have been proved to be important for the retainment of siRNA silence activity. Considering the importance of the middle site and the two terminals for the oligonucleotides, we design the amino-isonucleoside modified antisense oligonucleotides II–VII and amino-isonucleoside modified siRNAs 2–7. We analyzed the physicochemical properties of such modifications at different positions of ASONs and further investigated the RNAi potency of each strand of the modified siRNAs (related to cyclin B1-complexed CDC2). These results will be useful in the choice of modified strategies.

2 Materials and method

2.1 General

All solvents were dried and distilled prior to use. Chemical reagents were purchased from Acros and Sigma Co. Thin layer chromatography was performed on silica gel GF-254 (Qing-Dao Chemical Co., China) plates with detection by UV or by heating. Silica gel (200–300 mesh; Qing-Dao Chemical Co.) was used for short column chromatography. NMR spectra were recorded on a Varian VXR-300 and Varian Inova-500 instrument. ¹H NMR spectra were referenced using internal standard TMS and ³¹P NMR spectra using external standard 85% H₃PO₄. Mass spectra (ESI-TOF/MS) and high resolution mass spectra (ESI-TOF/HRMS) were obtained on MDS SCIEX QSTAR and Bruker DALTONICS APEX IV 70e instruments. MALDI-TOF mass spectra for oligonucleotides were obtained at SIMADZU AXIMA CFR plus, and the data are reported in m/z (intensity to 100%).

2.2 Synthesis of amino-isonucleoside phosphoramidites

4-Deoxy-4-(adenin-9-yl)-6-deoxy-6-(N-tert-butylxoycarbonyl)-amino-2,5-anhydro-L-mannitol (4) To a mixture of N-(Boc)glycine (0.95 g, 5.41 mmol) and DCC (1.29 g, 6.25 mmol) in CH₂Cl₂ (10 mL) were added isoA₁ (1.167 g, 4.16 mmol) and N,N-diisopropylethylamine (0.84 mL, 4.16 mmol) in anhydrous DMF (10 mL). The resulting mixture was stirred at room temperature for 48 h and was concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography (10:1 dichloromethane/methanol, 0.5% NH₃·H₂O) to yield compound 4 (0.751 g, recovery yield 63.6%).

¹H NMR (500 MHz, DMSO-d₆): 1.37 (s, 9H, Boc), 3.23–3.26 (t, 2H, H-6a, H-6b), 3.49–3.51 (t, 2H, BocNHCH₂), 3.54–3.59 (m, 1H, H-1a), 3.64–3.68 (m, 1H, H-1b), 3.81–3.84 (m, 1H, H-2), 4.44–4.47 (m, 1H, H-5), 4.50–4.54 (t, 1H, H-4), 4.73–4.77 (m, 1H, H-3), 4.84–4.86 (t, 1H, 1-0H), 5.61–5.62 (d, 1H, 3-OH), 6.90–6.92 (t, 1H, BocNH), 7.99 (t, 1H, 6-NH); For adenin-9-yl: 7.24 (s, 2H, –NH₃), 8.08 (s, 1H, H-8), 8.14 (s, 1H, H-2). ¹³C NMR (125 MHz, DMSO-d₆): 28.2 (Boc), 40.3 (C-6), 43.1 (BocNHCH₂), 61.4 (C-1), 64.0 (C-4), 72.8 (C-3), 76.9 (C-5), 78.0 (Boc), 83.3 (C-2), 155.8 (Boc), 169.7 (6-CO); For adenin-9-yl: 119.4 (C-5), 140.7 (C-8), 149.4 (C-4), 152.3 (C-2), 156.1 (C-6). HRMS (ESI-TOF): Anal. calcd for C₁₈H₂₇N₇O₆: 460.1917. Found: 460.1915.

4-Deoxy-4-(6-benzoylamino-purin-9-yl)-6-deoxy-6-(N-tert-butylxoycarbonyl)-amino-2,5-anhydro-L-mannitol (5) Compound 4 (1.10g, 2.51 mmol) was dissolved in dry pyridine (20 mL). TMSCl (3.8 mL, 25.11 mmol) was added at 0 °C. The resulting solution was stirred at room temperature for 3 h. Benzoyl chloride (1.93 mL, 15.07 mmol) was added at 0 °C and the solution was stirred at room temperature for 3 h. The pH of the solution was adjusted to 8–9 by NH₃·H₂O at 0 °C. The solution was stirred at room temperature for 1 h. After evaporation, the mixture was dissolved in CH₂Cl₂ (50 mL). After filtration, the solvent was evaporated under vacuum and the residue was purified by silica gel
column chromatography (18:1 dichloromethane/methanol, 0.2% NH₃·H₂O) to yield 5 (1.08 g, 79.4%).

3H NMR (500 MHz, CDCl₃): 1.39 (s, 9H, Boc), 3.40 (br s, 1H, H-6a), 3.53 (br s, 1H, H-6b), 3.76 (d, 2H, BocNH-CH₂), 3.85 (br s, 2H, H-1a, H-1b), 4.05–4.14 (br s, 2H, H-2, H-3), 4.66 (s, 1H, H-4), 5.06 (br s, 1H, H-3), 5.38 (br s, 1H, 1-OH), 5.65 (br s, 1H, 3-OH), 7.14 (br s, 1H, BocNH), 7.45–7.48 (t, 2H, Ph), 7.54–7.57 (t, 1H, Ph), 7.96–7.98 (d, 2H, Ph); For adenin-9-yl: 8.15 (s, 1H, H-8), 8.53 (s, 1H, H-2), 9.48 (br s, 1H, NHCOPh). 13C NMR (125 MHz, CDCl₃): 28.3 (Boc), 40.4 (C-6), 44.4 (BocNHCH₂), 62.1 (C-1), 65.3 (C-4), 73.8 (C-3), 77.7 (C-5), 80.5 (Boc), 83.5 (C-2), 128.0, 128.8, 132.9, 133.2 (Ph), 151.9 (Boc), 171.1 (6- CO); For adenin-9-yl: 123.1 (C-5), 144.0 (C-8), 149.3 (C-4), 151.5 (C-2), 156.5 (C-6), 165.2 (NHCOPh). MS (ESI-TOF)+: m/z 542 (M+H)+, 564(M+Na)+. Anal. calcd for C₂5H₃₁N₇O₇ (541.23): C, 55.19; H, 6.14; N, 18.10. Found: C, 55.7; H, 5.77; N, 18.10. 4-Deoxy-4-(6-benzoylamino-purin-9-yl)-6-deoxy-6-(N-trifluorooctacyl-glycin)-amino-2,5-anhydro-L-mannitol (6)

Compound 5 (0.82 g, 1.52 mmol) was dissolved in 3 M HCl-EtOAc (60 mL). The mixture was stirred for 4 h and neutralized by NaOH. Then the solution was concentrated in vacuo and the mixture was dissolved in EtOH (40 mL). After filtration, the solvent was evaporated under vacuum to yield the crude compound 4-deoxy-4-(6-benzoylamino-purin-9-yl)-6-deoxy-6-(N-glycin)-amino-2,5-anhydro-L-mannitol (6-1). Et₃N (1.34 mL, 9.12 mmol) and ethyl trifluoracetate (0.83 mL, 6.1 mmol) were added dropwise to the mixture of compound 6-1 (0.67 g, 1.52 mmol) and CH₂OH (20 mL) in an ice bath. The mixture was stirred overnight at room temperature. After evaporation, the residue was purified by silica gel chromatography (10:1 dichloromethane/methanol, 0.2% Et₃N) to yield 6 (0.79 g, colorless syrup) in 97.5% yield.

3H NMR (500 MHz, DMSO-d₆): 3.26–3.29 (m, 2H, H-6a, H-6b), 3.58–3.63 (m, 1H, H-1a), 3.68–3.72 (m, 1H, H-1b), 3.78–3.80 (m, 2H, TFANHCH₂), 3.87–3.90 (m, 1H, H-2), 4.54–4.58 (m, 1H, H-5), 4.68–4.72 (d, 4H, H-4), 4.78–4.83 (m, 1H, H-3), 4.88–4.91 (t, 1H, 1-OH), 5.73 (d, 1H, 3-OH), 7.54–7.57 (t, 2H, Ph), 7.64–7.67 (t, 1H, Ph), 8.04–8.06 (d, 2H, Ph), 8.33–8.35 (t, 1H, 6-NH), 9.59–9.61 (t, 1H, NHCOF); For adenin-9-yl: 8.43 (s, 1H, H-8), 8.75 (s, 1H, H-2), 11.17 (s, 1H, NHCOF). 13C NMR (125 MHz, DMSO-d₆): 40.5 (C-6), 41.7 (TFANHCH₂), 61.3 (C-1), 64.2 (C-4), 72.9 (C-3), 76.7 (C-5), 83.2 (C-2), 128.4, 132.4, 133.4 (Ph), 156.5, 156.8 (CF₃CO), 167.4 (6-CO); For adenin-9-yl: 126.0 (C-5), 144.3 (C-8), 150.3 (C-4), 151.3 (C-2), 152.3 (C-6), 165.6 (NHCOF). MS (ESI-TOF)+: m/z 538 (M+H)+, 560 (M+Na)+. HRMS (ESI-TOF)+: m/z 542 (M+H)+, 564 (M+Na)+. Anal. calcd for C₄₃H₄₀N₇O₈F₃ (840.2966, 862.2785, 878.2520; Found: 840.2966, 862.2785, 878.2520.

J-O-(4,4'-Dimethoxytrityl)-3-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite-4-deoxy-4-(6-benzoylamino-purin-9-yl)-6-deoxy-6-(N-trifluoroacetyl-glycin)-amino-2,5-anhydro-L-mannitol (2)

Compound 7 (380 mg, 0.453 mmol) and 1-H-tetrazole (42 mg, 0.602 mmol) were dissolved in anhydrous CH₂Cl₂ (10 mL) under argon atmosphere. The mixture was stirred at room temperature for about 15 min, 2-cyanoethyl-N,N'-tetraisopropyl-phosphoramidite (0.19 mL, 0.590 mmol) was added dropwise, and the reaction mixture was stirred for 3 h. TLC analysis (50:50 CH₂Cl₂/EtOAc) indicated completion of the reaction. The reaction mixture was then diluted with CH₂Cl₂ (20 mL), extracted with 5% NaHCO₃ solution (15 mL × 2), washed with brine (20 mL × 2), and dried over Na₂SO₄. Evaporation to dryness yielded a white foam, which was dissolved in CH₂Cl₂ (3 mL), and applied to a silica gel column equilibrated with CH₂Cl₂ containing 0.5% Et₃N. Elution with CH₂Cl₂/EtOAc (1:1–1:6, 0.5% Et₃N) yielded 2 (291 mg, 61.0%) as a white foam.

3P NMR (121.50 MHz, DMSO-d₆): 149.63 and 150.32 (d).

2.3 Synthesis of the amino-isonucleoside modified oligonucleotides

The amino-isonucleoside modified oligonucleotides were prepared by solid phase phosphoramidite chemistry (DMT
on) using an Applied Biosystems 381A DNA synthesizer. For the purpose of operation convenience, the synthesis was started with the commercially available controlled pore glass (CPG) with cytidine-loaded. The 0.1 M solution of amidites 1 and 2 in anhydrous acetonitrile was used for the synthesis of the modified oligonucleotides. For incorporation of 1 and 2, the phosphoramidite solutions were delivered in three portions, each followed by a 10 min coupling wait time. All other steps in the protocol provided by Applied Biosystems were used without modifications. After completion of the synthesis, CPG was suspended in aqueous ammonium hydroxide (30 wt%) and kept at 55 °C overnight to complete the removal of all protecting groups. The CPG was filtered and the crude oligonucleotides were purified by high performance liquid chromatography (HPLC, ZORBAX Bio Series Oligo Column, 6.2 mm ID × 80 mm), with gradient eluting using eluants A (MeCN/0.02 M NaH2PO4, 1:4) and B (1.0 M NaCl in A) at 1.0 mL/min flow rate. The fractions containing pure oligonucleotides were lyophilized and stored at −20 °C. Other natural oligonucleotides were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.

2.4 UV melting experiments

Determination of the $T_m$ of the ASON/DNA hybrids was carried out in the following buffer: 57 mM Tris-HCl (pH 7.5), 57 mM KCl, 1 mM MgCl2. Absorbance was monitored at 260 nm in the temperature range from 20 to 80 °C using a UV-visible spectrophotometer with the heating rate of 0.5 °C per minute. Prior to measurements, the samples (0.85 μM of ASON and 0.75 μM of DNA mixture) were preannealed by heating to 90 °C for 5 min followed by slow cooling to 4 °C and keeping this temperature overnight.

2.5 CD spectra

CD Spectra were recorded from 300 to 200 nm in 1 cm path length cuvettes. Spectra were obtained with an ASON/DNA duplex concentration of 0.75 μM in buffer containing 57 mM Tris-HCl (pH 7.5), 57 mM KCl and 1 mM MgCl2. All spectra were measured at 20 °C with a J715 CD spectrophotometer (JAC).

2.6 Exonuclease degradation studies

Stability of the ASONs toward 3′-exonuclease was tested using snake-venom phosphodiesterase (SVPDE). All reactions were performed at 7.1 μM DNA concentration in 56 mM Tris-HCl (pH 7.9) and 4.4 mM MgCl2 at 37 °C. Exonuclease concentration of 28.6 ng/μL was used for digestion of oligonucleotides. The total reaction volume was 14 μL. Aliquots were taken at 0, 5, 10, 20, 40, 60 min and quenched by addition of the same volume of 50 mM EDTA in 95% formamide. Reaction progress was monitored by 20% denaturing (7 M urea) PAGE and was visualized by staining with SYBR gold and quantified by Model & Storm 860 hardware and Imagequant software (Amersham Biosciences, PKU, China).

2.7 Endonuclease degradation studies

Stability of ASONs toward endonuclease was tested using DNase I from bovine pancreas. Reactions were carried out at 10 μM DNA concentration in 100 mM Tris-HCl (pH 7.5) and 10 mM MgCl2 at 37 °C using 15 unit of DNase I. The total reaction volume was 10 μL. Aliquots were taken at 0, 2, 4, 10, 20, 40 and 60 min and quenched with the same volume of 50 mM EDTA in 95% formamide, which were resolved in 20% polyacrylamide, respectively, then applying in denaturing (7 M urea) gel electrophoresis. The analytic results were visualized by staining with SYBR gold and quantified by Model & Storm 860 hardware and Imagequant software (Amersham Biosciences, PKU, China).

2.8 Computer simulation

All Molecular dynamics (MD) simulations were performed with the AMBER 8 molecular simulation package. The AMBER 99 force field was used to describe the DNA:RNA. Starting models of the studied DNA:RNA duplexes were built in the A canonical structures using the Insight II package. All constructed oligonucleotide duplexes were solvated in TIP3P water using a rectangular box, which extended 10 Å away from any solute atom. To neutralize the negative charges of simulated molecules, Na+ counterions were placed next to each phosphate group. Molecular dynamics (MD) simulations were carried out using the SANDER module of AMBER 8. The production simulations of 2.0 ns for all duplexes were performed at constant pressure (1 atm) and temperature (300 K). The final structure of each duplex was produced from 1000 steps of the minimized averaged structure of the last 1.5 ns of MD. Free-energy analysis was performed using the MM_PBSA scripts supplied by AMBER 8.

2.9 Cell culture

HEK293 cell line was obtained from Dr. Zicai Liang (LNAT, IMM, PKU). The HEK293 cell line was cultured in DMEM (Hyclon, USA) supplemented with 10% FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO2.
2.10 RNAi potency assay

Oligonucleotides and plasmids in RNAi assay: DNA oligonucleotides were from Invitrogen (Beijing, China). Normal RNA oligonucleotides were from Genepharma (Shanghai, China). Plasmid DNAs were extracted using a mini-purification kit (Promega). siQuant vector was a gift from Institute of Molecular Medicine, Peking University, Beijing.

Human embryonic kidney (HEK293) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Gibco). The cells were seeded into 24-well plates at a density of ~1 × 10⁵ cells/well one day before transfection. siQuant vector (0.17 µg/well) carrying the target site of tested siRNA was transfected into HEK293 cells at approximately 50% confluence, together with pRL-TK control vector (0.017 µg/well), with or without the siRNA (16.7 nM). The activities of both luciferases were determined by a fluorometer (Synergy HT, BioTek, USA) before the firefly luciferase activity was normalized to renilla luciferase for each well. Silencing efficiency of each siRNA was calculated by comparison with a sample without siRNA treatment. All experiments were performed in triplicate and repeated at least twice.

3 Results and discussion

3.1 Synthesis of building blocks 1, 2 and 3 for isoA₁, isoA₂ and isoT₁

The building blocks 1 and 3 (Figure 2) for isoA₁ and isoT₁ were synthesized following the previously reported protocol by Li et al. [18]. For the building block 2 of isoA₂ which has a longer side chain containing an amino group, the synthetic route was altered to include glycylation of 6-amino group, which requires a different protecting group of the glycol (Scheme 1).

3.2 Synthesis of oligonucleotides II–VII

Oligodeoxynucleotide synthesis was carried out at the 1 µmol scale using Applied Biosystem model 381 DNA Synthesizer according to regular phosphoramidite chemistry created by Caruthers. At the modified position, the native phosphoramidites (0.12 M) were replaced by corresponding amino-isonucleoside phosphoramidites building blocks 1 and 2 (0.1 M), respectively, characterized. The isolated yields (by anion exchange high-performance liquid chromatography) of modified oligonucleotides (II–VII) were 30%–40%, which are similar to those during unmodified DNA oligomer synthesis. The products were characterized by MALDI-TOF mass spectroscopy (Table 1).

3.3 Synthesis of siRNA single strand A or B

According to the standard procedure for the synthesis of single strand of RNA oligomer, the native phosphoramidites (0.12 M) were respectively replaced by corresponding amino-isonucleoside phosphoramidites building blocks 1, 2 and 3 (0.1 M) at the modified step. Furthermore, an extended coupling time of 900 second was used to replace the standard coupling time of 600 second used for the native phosphoramidites (rAbz, rGAc, rCAc, rU) due to the steric effect of modified phosphoramidites. The crude product was purified by anion exchange high-performance liquid chromatography and characterized by MALDI-TOF mass spectroscopy (Table 2).

3.4 Binding ability to complementary single strand DNA or RNA

The thermal stability of duplexes involving isoA₁ and isoA₂ was examined by hybridization properties of oligonucleotides I–VII with complementary DNA VIII as shown in Table 3. The results revealed that all oligomers II–VII succeeded in hybridizing with VIII to form stable duplexes. When isoA₁ and isoA₂ were incorporated in the center of oligomers (IV and VI), the Tₘ values were decreased by 5.8 and 7.9 °C, respectively, when compared with their natural counterpart. When isoA₁ and isoA₂ were appended to the 3′-end of oligomers (III and VII), the Tₘ values were decreased by 3.9 and 5.0 °C, respectively. When isoA₁ and isoA₂ were appended to the 5′-end of oligomers (II and V), the Tₘ values were decreased by 3.9 and 4.9 °C, respectively. The reason of such thermal stability decreasing is that the
formation of Watson-Crick hydrogen bonding was perturbed by the torsion of the backbone at the position of modification. Also, the thermal stability results of the duplexes involving isoA1 and isoA2 with complementary RNA IX are shown in Table 3, demonstrating that the $T_m$ values of the modified ones were decreasing when compared with their natural counterpart. Especially, the isoA2 incorporated ones (V, VI, and VII) show less influence than the isoA1 incorporated ones (II, III and IV). But this trend was not obvious in the DNA/DNA duplex. The structure of DNA/RNA duplex ($T_m$ = 76.0 °C) is more compact than the DNA/DNA (68.0 °C) because of the 2′-hydroxyl group of RNA. The difference between isoA1 and isoA2 modification may be due to the release of the torsion strain of duplex in the long side chain of amide in isoA2.

### Table 1 Modification on ASONs by isoA1 and isoA2 incorporation

| No. | Modification on ASON | MALDI-TOF MS (calcd) | MALDI-TOF MS (found) |
|-----|----------------------|-----------------------|-----------------------|
| I   | 5′-ACATCTCCGCGACTCCCCTC-3′ | 5933 | 5933 |
| II  | 5′-ACisoA1TCTCCGCGACTCCCCTC-3′ | 5933 | 5932 |
| III | 5′-ACATCTCCGCGACTCCCCTC-3′ | 5933 | 5933 |
| IV  | 5′-ACATCTCCGCGisoA1TCTCCGCG-3′ | 5990 | 5993 |
| V   | 5′-ACisoA2TCTCCGCGACTCCCCTC-3′ | 5990 | 5993 |
| VI  | 5′-ACATCTCCGCGisoA2TCTCCGCG-3′ | 5990 | 5993 |
| VII | 5′-ACATCTCCGCGACTCCCCTC-3′ | 5990 | 5993 |
| c-DNA (VIII) | 3′-TGTAGAGGCGTAGGAG-5′ | | |

### Table 2 Modified siCdc-2 by isoA1, isoA2 and isoT1 incorporation

| No. | Modification on single strands of siCdc2 | MALDI-TOF MS (calcd) | MALDI-TOF MS (found) |
|-----|-----------------------------------------|-----------------------|-----------------------|
| siCdc-2-A | 5′-UCGGGAAAUUUCUCUAUUA tt-3′ | | |
| siCdc-2-B | 3′-tt AGCCCUUUAAAGAGAUAAU-5′ | | |
| A strand modification | RNA-2-A | 5′-UCGGGAAAUUisoT1UCUCUAUUA tt-3′ | 6616.92 | 6616.93 |
| RNA-3-A | 5′-UCGGGAAAUUUCUCUAUisoT1A tt-3′ | 6616.00 | | |
| B strand modification | RNA-4-B | 3′-tt AGCCCUUUAAAGAGAUAAU-5′ | 6671.98 | 6672.39 |
| RNA-5-B | 3′-tt AGCCCUUUAAisoA1AGAGAUAAU-5′ | 6671.98 | 6671.48 |
| RNA-6-B | 3′-tt AGCCCUUUAAisoA2AGAGAUAAU-5′ | 6729.00 | 6730.53 |
| RNA-7-B | 3′-tt AGCCCUUUAAisoA2AGAGAUAAU-5′ | 6729.00 | 6729.66 |

### Table 3 $T_m$ values of duplex and calculated free energy (G) of DNA/RNA by computer simulation

| DNA/DNA duplex | $T_m$ (°C) | DNA/DNA | $\Delta T_m$ (°C) | DNA/RNA duplex | $T_m$ (°C) | DNA/RNA | $\Delta T_m$ (°C) | G (kcal/mol) | DNA/RNA |
|----------------|------------|---------|------------------|----------------|------------|---------|------------------|-------------|---------|
| I/VIII | 68.0 | – | I/IX | 76.0 | – | – | –55.45 |
| II/VIII | 62.2 | –5.8 | II/IX | 71.0 | –5.0 | – | –44.76 |
| III/VIII | 64.1 | –3.9 | III/IX | 70.8 | –5.2 | –49.55 |
| IV/VIII | 64.1 | –3.9 | IV/IX | 71.6 | –4.4 | –54.12 |
| V/VIII | 60.1 | –7.9 | V/IX | 72.5 | –3.5 | – | –46.52 |
| VI/VIII | 63.1 | –4.9 | VI/IX | 72.5 | –3.5 | –45.27 |
| VII/VIII | 63.0 | –5.0 | VII/IX | 72.5 | –3.5 | –51.60 |

a) Compare to $T_m$ value (68.0 °C) of I/VIII duplex; b) compare to $T_m$ value (76.0 °C) of I/IX duplex.
3.5 Circular dichroism

To further confirm the conformations of the hybrid duplexes formed from II–VII with their complementary VIII, the circular dichroism (CD) spectra were performed (Figure 3). The results showed that the modified duplexes (II/VIII–VII/VIII) possessed very similar conformations to that of I/VIII, i.e. the standard B-form helix. Although the presence of one incorporated amino-isonucleotide isoA1 or isoA2 twisted the backbone, the global conformation could not be significantly affected.

3.6 Computer simulations for DNA/RNA duplexes

The hydrogen bonding was broken at the position of isonucleoside in V/IX and VI/IX, but the one in VII/IX wasn’t. The amide at the 6-position of the sugar ring in isoA2 was involved in the formation of the hydrogen bonding at the position of isonucleoside in VII/IX. The reason is that self-regulation releases torsion strain of duplex, resulting in a lower free energy in VII/IX.

Both isoA1 inserted ASONs (II–IV) can form intact hydrogen bonding with IX (Figure 4). But in isoA2 inserted ones (V–VII), different phenomena were observed (Figure 5). Compared to VII/IX, the hydrogen bonding was broken at the position of isonucleoside in VI/IX, VII/IX. This could be the reason why V/IX and VI/IX have a higher free energy than VII/IX, although their $T_m$ values are the same, as perhaps the amide at the 6-position of the sugar ring in isoA2 is involved in the formation of the hydrogen bonding at the position of isonucleoside. This may be due to the self regulation that releases torsion strain of duplex.

3.7 RNAi potency of siRNAs

To evaluate the influences on the gene silencing efficiencies of both strands of amino-isonucleoside modified siRNA, two mRNA targets were constructed for the both strands of siCdc2 via siQuant vector, respectively. Each strand of siCdc2 is considerably active toward its target mRNA in the artificial RNAi assay model, in which an mRNA fragment, perfectly matching the A strand or B strand of siCdc2, was

| DNA Duplex | Positive cotton peak (nm) | Negative cotton peak (nm) |
|------------|----------------------------|--------------------------|
| I/VIII     | 254                        | 220                      |
| II/VIII    | 265                        | 213                      |
| III/VIII   | 267                        | 213                      |
| IV/VIII    | 266                        | 215                      |
| V/VIII     | 266                        | 216                      |
| VI/VIII    | 268                        | 213                      |
| VII/VIII   | 270                        | 214                      |

Figure 3 CD spectra of I–VII with their complementary DNA VIII (nm). CD buffer: 57 mM Tris-HCl (pH 7.5), 57 mM KCl and 1 mM MgCl2.

Figure 4 Computer simulated conformation of I/IX (first, left), III/IX (second, left), II/IX (second, right) and IV/IX (first, right).

Figure 5 Computer simulated conformation of VI/IX (left), V/IX (middle) and VII/IX (right). The hydrogen bonding was substituted by amide group at the position of isoA2 in V/IX, VI/IX.
fused in-frame with the firefly luciferase gene in a mammalian expression vector [19]. The resulting fusion reporter was then used as an artificial target. The siCdc2 (siRNA-1) and siRNA-2–siRNA-7 were examined for their silencing efficacy on the two targets (Figures 6 and 7).

![Figure 6](image)

**Figure 6** Gene silencing efficiencies of modified siCdc-2 for B strand as guide strand.

![Figure 7](image)

**Figure 7** Gene silencing efficiencies of modified siCdc-2 for A strand as guide strand.

We have reported the synthesis of isoA1 incorporated siRNA, and found that modification on the sense strand retained the silence efficiencies of the siRNA(Wee1) antisense strand [18]. Additionally, the conformation of an ASON was changed at the position of amino-isonucleotide incorporated, which would make the modified ASON less recognized by nucleases and increase its stability toward various enzymes. The thermal stability of modified siCdc2 duplex with amino-isonucleoside decreased with lower Tm values. Alternations in torsion angles could promote the passenger strand cleavage or separation from RISC, which could potentially increase silencing efficiency [18]. Here we continued to investigate the RNAi potency of amino-isonucleosides isoA1, isoA2 and isoT1 incorporated in the middle or 3’-end of siRNA A strand or B strand. It’s known that each strand of the siRNA can be the guide strand, which is loaded into RISC and executes the knockdown effect for the target itself. The gene silencing results of A strand will represent the off-target effect by sense strand loading into RISC, and the gene silencing results of B strand will represent real RNAi potency of siCdc-2. So the artificial assay system is used to investigate this phenomenon.

To investigate the potency of B strand, the A strand incorporated with isoT1 in the middle (RNA-2) and at the position close to 3’-end (RNA-3) were synthesized, respectively. While the former had no influence, the latter slightly increased the silence efficiency of B strand. As shown in the Figure 6 the siRNA-3 B-strand potency increased slightly. While in the Figure 7, the siRNA-3 A strand has no activity. We could image that both strands of siRNA can be loaded into RISC, so there is competition effect between them. Decreasing the activity of one strand may increase the other one. As previously reported, modification incorporated in the middle of A strand will inactivate the siRNA (RNA-4, 5, 6, 7) [20]. In the process of Ago2 recognition and sense strand release or cleavage, neither of the amino-isonucleoside modifications were suitable substrates for the protein. Because the longer side chain of isoA2 relieves torsion strain of the duplex (unpublished data), it may slightly enhance the release ability of the sense strand when compared with that of isoA1 modification. The side chains of the amides (RNA-6, RNA-7) have less influence than the amino ones (RNA-4, RNA-5), although both amino-isonucleoside modifications decreased RNAi potency when incorporated in the middle of the B strands (Figure 6, Table 4).

In the previous reports, the 3’-end of the sense strand modified by 2’-OMe nucleosides and many others maintained the silencing efficiencies of siRNAs [21]. Our results show that the overall influence on the RNAi efficiency of

| No. | siCdc-2 | Modified position | Silencing efficiencies of B strand (%) | Silencing efficiencies of A strand (%) |
|-----|---------|-------------------|----------------------------------------|----------------------------------------|
| RNA-1 | A: 5’-UCG GGA AAU UUC UCU AUU Att-3’ B: 3’-nt AGC CCU UUA AAG AGA UAA U-5’ | | 94.80 | 79.50 |
| RNA-2 | 5’-UCG GGA AAU isoT1 UUC UCU AUU Att-3’B | 10 (S) | 93.10 | 27.10 |
| RNA-3 | 5’-UCG GGA AAU UUC UCU AUU isoT1 Att -3’B | 18 (S) | 96.50 | 5.50 |
| RNA-4 | A/3’-nt AGC CCU UU isoA2 AAG AGA UAA U-5’ | 11 (As) | 39.00 | 71.60 |
| RNA-5 | A/3’-nt A GC CCU UUA isoA2 AGA UAA U-5’ | 10 (As) | 11.00 | 70.80 |
| RNA-6 | A/3’-nt A GC CCU UUA isoA2 AGA UAA U-5’ | 10 (As) | 46.50 | 68.90 |
| RNA-7 | A/3’-nt A GC CCU UU isoA2 AAG AGA UAA U-5’ | 11 (As) | 43.00 | 80.40 |
modified A strand is similar to that of B strand. However, because the original RNAi potency of A strand is relatively low, the influence of modifications is more obvious [22]. When isoT1 was incorporated in the A strand at the 18th position, its RNAi efficiency completely disappeared (RNA-3) (Figure 7, Table 4). It’s reported that the PAZ domain of Ago2 initially binds to the 3’-end of siRNA, and once the target mRNA comes and starts to anneal to the siRNA, the 3’-end of the guide strand is released from it, with the release step being the rate-limiting step in this process [23]. The amino-isonucleosides with greater stereo hindrance than the 2′-OMe nucleosides incorporated near the 3’-end of the guide strand may interrupt the binding or releasing step. The possibility of difficulty in the TRBP or Dicer binding step before the formation of the RISC loading complex (RLC) cannot be eliminated, for TRBP binds 3’-half of siRNA and then transfers it to Ago2 to form RLC under the help of Dicer. The steric hindrance of isonucleosides may influence protein recognition and binding process in the prior step.

Combined with our previous reports [18], it is concluded that amino-isonucleosides incorporated in the middle, 3’-end or 5’-end of siRNA A strand all inhibit the RNAi efficiency of this strand, which means reduction of the off-target effect by sense strand loading into RISC. Additionally, these two reporters can be treated as two independent siRNA targets with different potency. Modification in one strand merely influenced the silencing efficiency of another, which would mean the modifications didn’t influence the cleavage or unwinding of the modified passenger strand of siRNA after RISC formation. In fact, it is necessary to modify more other positions of passenger strand of siRNA to give more detailed data to support the conclusion above in the future work. Both strands of siRNA can be loaded into RISC and execute knockdown effect of the corresponding target, which may be due to the competitive effect between A strand and B strand loading into RISC [24].

4 Conclusion

In this work, we have reported the synthesis of a novel class of amino-isonucleoside isoA2. Furthermore, two amino-isonucleosides isoA1 and isoA2 were selected to incorporate into the natural ASOn I to investigate the basic physicochemical properties. The UV melting experiment and the CD spectra showed that individual isoA1 and isoA2 modifications slightly affect the stability and conformation of duplexes, and III and VII were highly resistant to SVPDE, meanwhile II was slightly resistant to the exonuclease.

We further incorporated these amino-isonucleosides, including isoT1, into a siRNA (siCdc-2). Especially when isonucleoside was incorporated at the 3’-end of A strand, the RNAi efficiency of B strand was slightly increased. Meanwhile, amino-isonucleosides incorporated in the middle, 3’-end or 5’-end of siRNA sense strand all decrease the RNAi potency of this strand. Those results mean that the off-target effect by sense strand loading into RISC could be reduced by sense strand modification with amino-isonucleoside. We have obtained some promising results from amino-isonucleosides incorporation with ASOns or siRNAs, which could make some contributions to further research and therapeutic application.

We thank Prof. LIANG Zi-Cai, DU Quan and YI Fan in Peking University, for providing siRNA sequence and assistance with biological assay experiments. This work was supported by the National Natural Science Foundation of China (20932001), and the Ministry of Science and Technology of China (2006AA02Z144, 2009ZX09503).

Abbreviations

ASOns antisense oligonucleotides
CD circular dichroism
CPG controlled pore glass
DCO dicyclohexyl carbodiimide
DIPEA diisopropylethylamine
DMF dimethyl formide
DMSO dimethylsulfoxide
DMT 4,4’-dimethoxytriphenyl
EDTA ethylenediamine tetraacetic acid
ESI-TOF electrospray ionization-time of flight
HRMS high resolution mass spectrometry
HPLC high performance liquid chromatography
MALDI matrix-assisted laser desorption ionization
MS mass spectrometry
PNA peptide nucleic acid
Py pyridine
RNAi RNA interference
RISC RNA induced silencing complex
RLC RISC loading complex
SVPDE snake venom phosphodiesterase
TEAB triethyl-ammonium bicarbonate
TFA trifluoroacetyl
THF tetrahydrofuran
TIP3P transferable intermolecular potential (function) 3 points
Tm temperature of melting
TMSCl trimethylsilyl chloride
TOF time of flying

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