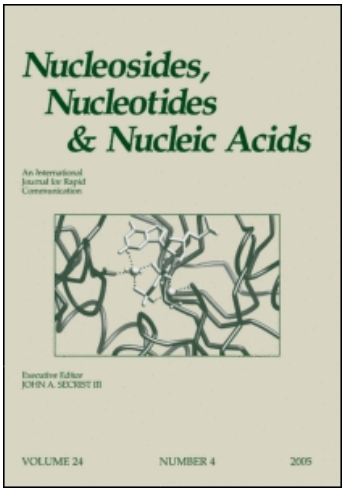

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Comparison Between Properties Of 2'-*O*,4'-*C*-Ethylene-Bridged Nucleic Acid (Ena®) Phosphorothioate Oligonucleotides And N3'-P5' Thiophosphoramidate Oligonucleotides

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COMPARISON BETWEEN PROPERTIES OF 2′-*O*,4′-*C*-ETHYLENE-BRIDGED NUCLEIC ACID (ENA®) PHOSPHOROTHIOATE OLIGONUCLEOTIDES AND N3′-P5′ THIOPHOSPHORAMIDATE OLIGONUCLEOTIDES

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□ *Synthesis and properties of an oligonucleotide uniformly modified with 2′-O,4-C-ethylene-bridged nucleic acid (ENA) units were compared with those of GRN163, which is modified with N3′-P5′ thiophosphoramidates, with the sequence targeting human telomerase RNA subunit. Although an ENA phosphorothioate oligonucleotide, ENA-13, could be synthesized using ENA phosphoramidites on a 100-mg scale, synthesis of GRN163 was very hard even on a 1-μmol scale. In view of both stability of the duplex formation with complementary RNA and the efficiency of cellular uptake by endocytosis, ENA-13 was superior to GRN163. These findings suggest that ENA-13 has useful properties for antisense therapeutic application.*

Keywords ENA; NPS; CD; T_m; Cellular uptake

INTRODUCTION

Synthetic oligonucleotides are applied to various technologies such as gene expression analysis arrays and regulation of gene expression.[1,2] A large variety of modified oligonucleotides have been designed and synthesized to improve their affinity to targeted nucleic acids and their stability under application conditions such as culture medium and animal testing.[3,4]

Recently, we reported the synthesis of novel 2′-*O*,4′-*C*-ethylene nucleic acids (ENA; a in Figure 1) that have a less-strained six-membered ring[5,6] than the five-membered ring of 2′-*O*,4′-*C*-methylene nucleic acids,

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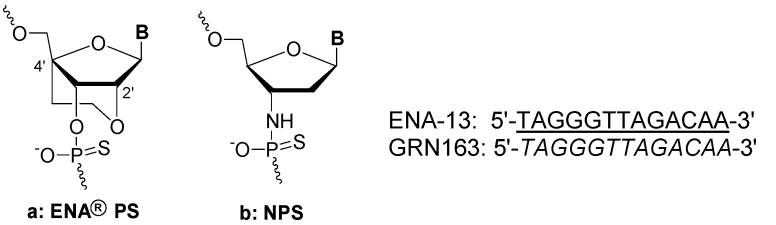


FIGURE 1 Structures of (**a**) 2′-*O*,4′-*C*-ethylene-bridged nucleic acids phosphorothioate (ENA PS) modification and (**b**) N3′-P5′ thiophosphoramidate (NPS) modification. ENA PS and NPS modifications are underlined and in italic, respectively. C indicates a 5-methylcytidine derivative.

named 2′,4′-BNA or LNA.[7–9] Both the sugar puckering of ENA and 2′,4′-BNA/LNA are locked in the *N*-conformation. The oligonucleotides with ENA residues exhibit a binding affinity to ssDNA and ssRNA as high as that of 2′,4′-BNA/LNA and show excellent triplex formation with dsDNA.[5,6,10] The ENA oligonucleotides also exhibit much higher nuclease resistance than the 2′,4′-BNA/LNA oligonucleotides.[5,6,10] We have shown the intracellular antisense activity of ENA oligonucleotides for vascular endothelial growth factor (VEGF) and organic anion transporting polypeptide (oatp) as targets.[11,12] The ENA oligonucleotides could be used for exon skipping to bind dystrophin pre-mRNA and inhibit its splicing reaction;[13,14] therefore, they are considered to be candidates for the next generation of antisense molecules.[4]

Telomerase is responsible for maintaining telomeres and is a riboprotein reverse transcriptase that catalyzes the addition of d(TTAGGG) on telomeres.[15] Its RNA subunit is utilized as a template for reverse transcription. Telomerase activity is detected in various human tumors, but not in most somatic cells.[16] Therefore, it is thought that telomerase is related to tumor proliferation and thus is an attractive target for cancer chemotherapy. It has been reported that some modified oligonucleotides become telomerase inhibitors such as peptide nucleic acids (PNA),[17] 2′-*O*-methyl RNA,[17] 2′-*O*-methoxyethyl RNA,[18] 2′,4′-BNA/LNA[19] and N3′-P5′ thiophosphoramidates[20] (NPS, **b** in Figure 1). In particular, an oligonucleotide modified with NPS, GRN163, which has 13 nucleotides complementary to the telomerase template region, showed high telomerase inhibitory activity in vitro and anticancer activity in animal models.[20–23] A lipid-modified NPS oligonucleotide (GRN163L) has entered phase I clinical trial.[24]

There are other reports of comparison of antisense activities with 2′-*O*-methyl RNA and NPS modification, in which NPS modification was found to be superior to 2′-*O*-methyl RNA.[25–26] In the present study, we compare the properties of GRN163 and the ENA phosphorothioate (PS) oligonucleotide with the same sequence as that of GRN163, in terms of synthesis, CD spectra, melting temperature, cellular uptake, and acidic stability for further antisense application.

MATERIALS AND METHODS

Synthesis of Oligonucleotides

2′-*O*,4′-*C*-Ethylene nucleoside-3′-*O*-phosphoramidite units were prepared according to reported methods.[5,6,27] Ethylene glycol-modified controlled pore grass (CPG) (**1**) was synthesized according to reported methods.[28] A uniformly modified ENA PS oligonucleotide, ENA-13, was prepared on an ABI 394 DNA/RNA synthesizer on a 1-μmol scale synthesis.[5,6] To obtain PS bonds, CPG was treated with 0.02 M xanthane hydride in CH_3CN:pyridine (9:1 v/v) solution for 900 s. After cleavage from the CPG and deprotection by treatment with aqueous ammonia for 5 h at 55°C, the ENA PS oligonucleotide was purified by a reverse-phase (RP) column (Cosmosil 75C_{18}-prep, 30 × 250 mm, Nakalai Tesque, Japan) with a solution of CH_3CN and 50 mM triethylammonium bicarbonate (pH 7.5). Deprotection of the 4, 4′-dimethoxytrityl group of the oligonucleotide was conducted by treatment with 80% acetic acid for 20 min. The purified oligonucleotide was analyzed by RP HPLC (column: Chromolith Performance RP-18e [4.6 × 100 mm, Merck], solvent A: 5% CH_3CN, 0.1 M triethylammonium acetate [TEAA, pH 7.0], solvent B: CH_3CN, column temperature: 60°C) and anion-exchange (AE) HPLC (column: TSKgel DEAE-5PW [6 × 50 mm, Tosoh], solvent A: 20% CH_3CN, solvent B: 20% CH_3CN, 67 mM sodium phosphate buffer [pH 6.8], 1.5 M KBr, column temperature: 60°C). Isolated yield of ENA-13 on a 1-μmol scale synthesis was 26 A_{260} units.

To synthesize ENA-13 on a semi-large scale, four columns each containing 26.5 μmol of ethylene glycol-modified CPG (**1**) were used. Isolated yield of ENA-13 on this scale synthesis was ca. 3300 A_{260} units (185 mg, isolated yield: 35%).

3′-*N*-Trityl-*N*6-benzoyl-3′-amino-2′,3′-dideoxyadenosine-5′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) and 3′-*N*-trityl-*N*2-isobutyryl-3′-amino-2′, 3′-dideoxyguanosine-5′-*O*-(2-cyanoethyl *N,N*-diisopropylphos-phoramidite) were purchased from Annovis Inc. 3′-*N*-Trityl-*N*4-benzoyl-3′-amino-2′, 3′-dideoxy-5-methylcytidine-5′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) and 3′-*N*-trityl-3′-amino-3′-deoxythymidine-5′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) were prepared according to reported methods.[20,29] GRN163 was synthesized according to reported methods.[20,29] To obtain PS bonds, CPG was treated with 15% S_8 in 1% triethylamine/ CH_3CN solution, 1% (w/v) 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) in CH_3CN solution or 0.02 M xanthane hydride in CH_3CN:pyridine (9:1 v/v) solution for 900 s. When xanthane hydride/CH_3CN:pyridine solution, which was the most effective reagent, was used, isolated yield of GRN163 on a 1-μmol scale synthesis was ca. 4 A_{260} units and isolated yield of GRN163 on a 30 μmol scale was 80 A_{260} units.

5′-Fluorescein-labeled ENA-13, 5′-FAM-ENA-13, and 3′-fluorescein-labeled GRN163, 3′-FAM-GRN163, were obtained using 6-FAM amidite (Applied Biosystems) at the final coupling step.

The structures of the modified oligonucleotides were determined by negative-ion ESI mass spectroscopy. ENA-13 calcd: 4916.10, found: 4916.09. GRN163 calcd: 4216.71, found: 4215.81. 5′-FAM-ENA-13 calcd: 5469.63, found: 5469.15. 3′-FAM-GRN163 calcd: 4770.22, found: 4769.67.

Measurement of Circular Dichroism (CD) Spectra

GRN163 or ENA-13 (2 μM) was dissolved in a buffer containing 10 mM sodium phosphate (pH 7.2), heated at 95°C for 10 min, and then cooled down to room temperature. The CD spectra of ENA-13 and GRN163 were obtained with a JASCO J-500C spectropolarimeter.

UV Melting Analysis

Each modified oligonucleotide (GRN163 or ENA-13) and an RNA oligonucleotide having hTR template sequences (5′-UUG UCU AAC CCT A-3′) at final concentrations of 3.4 μM were dissolved in a buffer containing 10 mM sodium phosphate (pH 7.2) and heated at 95°C for 10 min, and then cooled down to room temperature. The melting temperature (T_m) of each complex was measured with a UV spectrometer (UV-3100PC, Shimadzu, Japan) equipped with an incubator (Haake FE2, EKO) and a thermometer (SATO SK-1250MC) for temperature control.

Fluorescence Studies

DU-145 cells were incubated with 3′-FAM-GRN163 or 5′-FAM-ENA-13 (0.1, 1, or 10 μM) without cationic lipid for 24 h. Cells were removed, washed with PBS twice, and analyzed using flow cytometry (Beckman Coulter, EPICS XL—MCL System II).

Measurement of Stability of Oligonucleotides under Acidic Conditions

ENA-13 and GRN163 (7.2 μM) were each incubated in 20 mM sodium acetate (pH 5.0) at 37°C. Aliquots of each mixture were added to sodium borate buffer (pH 9.18) for neutralization at various time points and analyzed using AE HPLC (column: TSKgel DEAE-5PW [6 × 50 mm, Tosoh], solvent A: 20% CH_3CN, solvent B: 20% CH_3CN, 67 mM sodium phosphate buffer [pH 6.8], 1.5 M KBr, B% 20–70% [10 min], column temperature: 60°C). The percentage peak areas of ENA-13 and GRN163 at each time point were obtained by calculation from their initial respective areas. The percentage versus time values are plotted in Figure 2C.

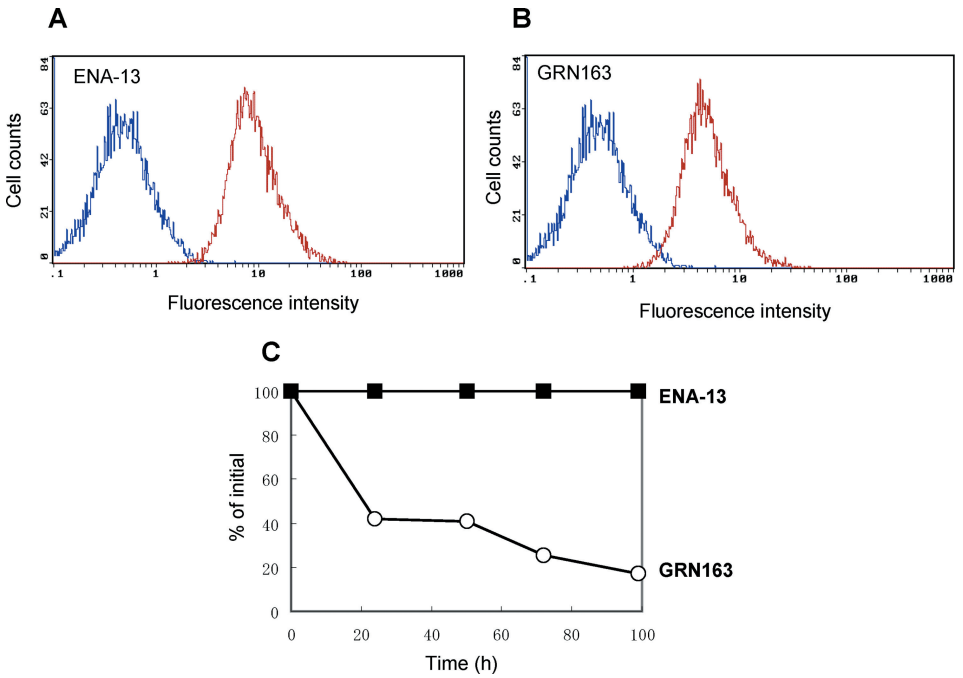


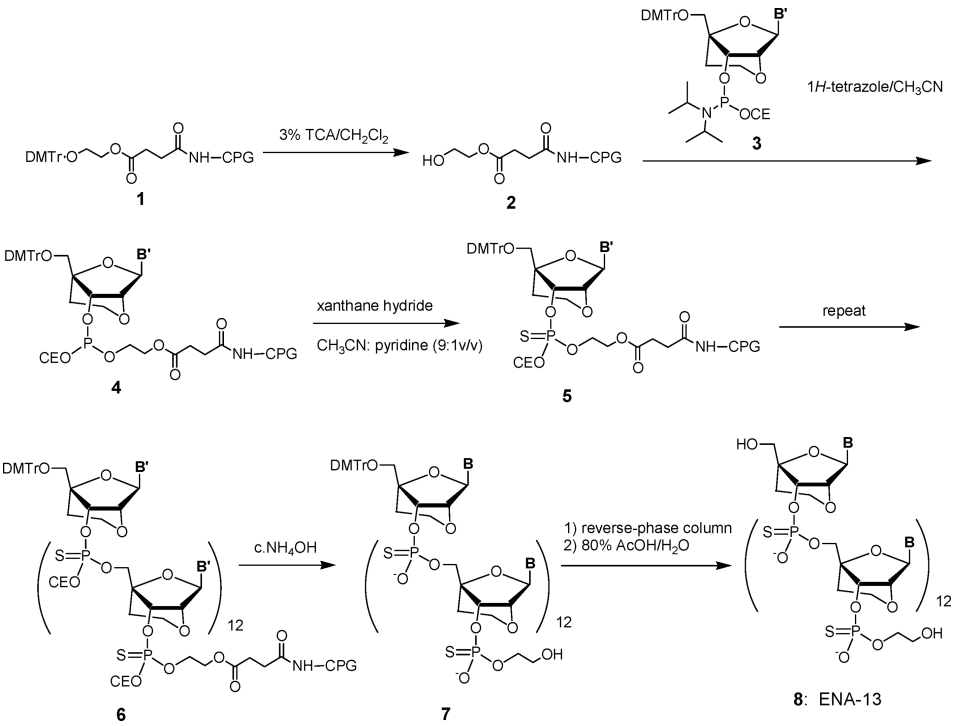
FIGURE 2 Cellular uptake of (A) 5′-FAM-ENA-13 and (B) 3′-FAM-GRN163. Left curves in each figure show control cells without labeled oligonucleotide. Right curves in each figure indicate cellular uptake of labeled oligonucleotides. Oligonucleotide concentration: 1 μM. (C) Stability of ENA-13 (closed squares) and GRN163 (open circles) under acidic solutions (pH 5.0). Oligonucleotide concentration: 7.2 μM; buffer: 20 mM sodium acetate (pH 5.0); temperature: 37°C.

RESULTS AND DISCUSSION

Synthesis of ENA-13 and GRN163

We have reported the synthesis of mixmers and gapmers of ENA/DNA and ENA/2′-*O*-Me RNA oligonucleotides.[5,6,11–14] A uniformly modified ENA PS oligonucleotide, ENA-13 (**8**), was prepared according to reported methods on a 1-μmol scale synthesis except for the thioation step (Scheme 1).[5,6] Although Beaucage reagent, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide, is generally used for introducing PS bonds into DNA oligonucleotides,[30] this reagent is not stable. As a stable reagent for synthesis of PS linkages, xanthane hydride has been utilized.[31] As shown in Scheme 1, CPG (**4**) was treated with 0.02 M xanthane hydride in CH_3CN:pyridine (9:1 v/v) solution for 900 s to give CPG (**5**) with the PS bond in the same good yield as in the case of Beaucage reagent. After the desired chain elongation using a DNA/RNA synthesizer, ammonia treatment, RP column purification, and 80% acetic acid treatment were conducted to obtain 26 A_{260} units of ENA-13.

For future chemical and biological testing, we tried to synthesize ENA oligonucleotides on a semi-large scale using a modified standard operation



SCHEME 1 Synthesis of ENA-13. B = nucleobase; B′ = protected nucleobase; CPG: controlled pore glass, CE: 2-cyanoethyl, DMTr: 4, 4′-dimethoxytrityl, TCA: trichloroacetic acid.

protocol for an ABI 394 DNA/RNA synthesizer known as a general-purpose DNA synthesizer. To obtain ENA-13 on a 100-mg scale, four columns each containing 26.5 μmol of ethylene glycol–modified CPG (**1**) were used (Scheme 1).[28] Although about 20 equivalents of ENA phosphoramidite (**3**) are normally used for coupling on a 1-μmol scale,[5,6] the amount of ENA phosphoramidite (**3**) was reduced to about 2 equivalents for coupling in the presence of about 10 equivalents of 1*H*-tetrazole for this semi-large scale. The coupling time of 0.1 M ENA phosphoramidite was 900 s, which is longer than in the case of normal DNA phosphoramidites. After the desired chain elongation, deprotection and purification were conducted to obtain 185 mg of ENA-13. Figures 3A and 3B show the RP and AE HPLC profiles of isolated ENA-13, respectively. ENA-13 showed 91% and 87% purity in the RP and AE HPLC analyses, respectively, and fairly good purity for the chemical evaluation and future biological testing.

On the other hand, when GRN163 was synthesized using phosphoramidite chemistry according to reported methods,[20,29] we observed large amounts of shorter products in the AE HPLC than that we desired. The isolated yields of GRN163 on the 1-μmol and 30-μmol scales were lower than those of ENA-13 (see Materials and Methods). This might be due to NPS bonds being unstable under acidic conditions in the deprotection step of

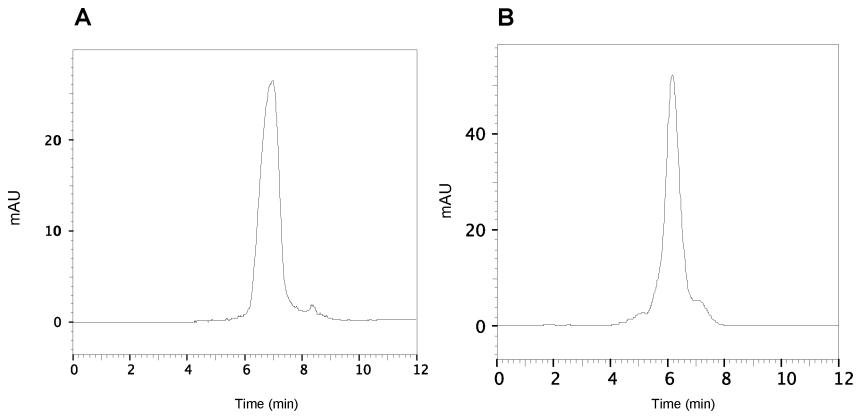


FIGURE 3 HPLC profiles of scale-up-synthesized ENA-13. (A) Reverse-phase HPLC analysis of ENA-13 (column: Chromolith Performance RP-18e [4.6 × 100 mm], solvent A: 5% CH_3CN, 0.1 M TEAA [pH 7.0], solvent B: CH_3CN, B%: 0–50% [10 min], flow rate: 2 mL/min, temperature: 60°C). (B) Anion-exchange HPLC analysis of ENA-13 (column: TSKgel DEAE-5PW [6 × 50 mm], solvent A: 20% CH_3CN, solvent B: 20% CH_3CN, 67 mM sodium phosphate buffer [pH 6.8], 1.5 M KBr, B%: 10–80% [8 min], flow rate: 2 mL/min, temperature: 60°C).

the trityl groups and/or that coupling yields of the phosphoramidite units are low. These results suggest that an improved method is necessary to synthesize GRN163 in good yield.

CD Spectra of ENA-13 and GRN163

ENA-13 and GRN163 contain contiguous guanine sequences which may form a G-quadruplex. Once oligonucleotides form thermodymamically stable G-quadruplexes, the oligonucleotides are not feasible for binding to hTR as antisense molecules. It has been reported that a 2′,4′-BNA/LNA oligonucleotide having a TGGGT sequence could form a stable G-quadruplex.[32]

To investigate the possibility of G-quadruplex formation of ENA-13 and GRN163, the CD spectra of ENA-13 and GRN163 were measured. It has been reported that guanine-rich oligonucleotides show specific CD spectra derived from parallel G-quadruplexes with a positive band at 260 nm and a negative band at 245 nm or anti-parallel G-quadruplexes with a positive band at 295 nm and a negative band at 265 nm.[28,33,34] Figures 4A and 4B show the CD spectra of ENA-13 and GRN163, exhibiting positive bands at around 271 nm and 275 nm, respectively. These values were not comparable to the previously reported data of parallel and anti-parallel G-quadruplexes.[33,34]

When the CD spectra of these oligonucleotides were measured at various increased temperatures, although the $[\theta]_{275}$ molar elliptic values of GRN163 decreased in accordance with increased temperatures (Figure 4B), probably due to the collapse of the base stacking, the $[\theta]_{271}$ molar elliptic values of ENA-13 were slightly increased with the λ_{max} of the spectra shifted from

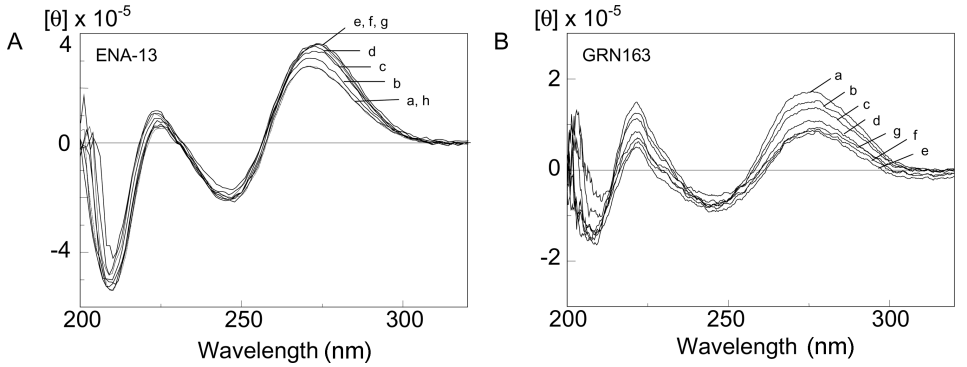


FIGURE 4 (A) CD spectra of ENA-13. Oligonucleotide concentration: 2 μM; buffer: PBS (pH 7.2). (B) CD spectra of GRN163. Oligonucleotide concentration: 2 μM; buffer: PBS (pH 7.2); temperature: a: 20°C, b: 30°C, c: 40°C, d: 50°C, e: 60°C, f: 70°C, g: 80°C, h: 80°C $\rightarrow$ 20°C.

271 nm to 274 nm (Figure 4A). Although the structure with the λ_{max} value at 271 nm appears to have been unstable and collapsed at high temperature, the structure with the λ_{max} value at 274 nm appears to have been stable. A single-stranded oligonucleotide fully modified with 2′,4′-BNA/LNA, even at a high temperature, also shows very stable CD melting spectra,[9] similar to those of ENA-13. These observations may be characteristic of single-stranded ENA and 2′,4′-BNA/LNA oligonucleotides derived from the rigid sugar conformation.

Melting Temperatures of ENA-13 and GRN163

GRN163 has been evaluated in a biochemical assay as a telomerase inhibitor, and the melting temperature (T_m) for the duplex formation with the complementary RNA strand was determined.[20,21] It has been reported that the T_m value of GRN163 in PBS was 70°C, which was higher than that of the isosequential natural oligodeoxynucleotide ($\Delta T_m = 24$°C).[20]

To compare the affinity of GRN163 for the target RNA with that of ENA-13, we formed their duplexes with an RNA oligonucleotide having an hTR target sequence, and their T_m values were measured under low salt conditions such as 10 mM sodium phosphate buffer (pH 7.2), because the T_m value of ENA-13 in PBS was considered too high to measure on the basis of previous reports (ΔT_m/ENA modification = +3 to 5°C).[5,6] The melting profiles revealed T_m values of 85°C and 51°C for the duplexes of ENA-13 and GRN163, respectively (Figure 5). These results clearly indicate that ENA-13 can form a more stable duplex than that of GRN163.

Cellular Uptake and Acidic Stability of ENA-13 and GRN163

It is reported that GRN163 shows excellent uptake into cells without an uptake enhancer such as lipids.[23] To compare cellular uptake between

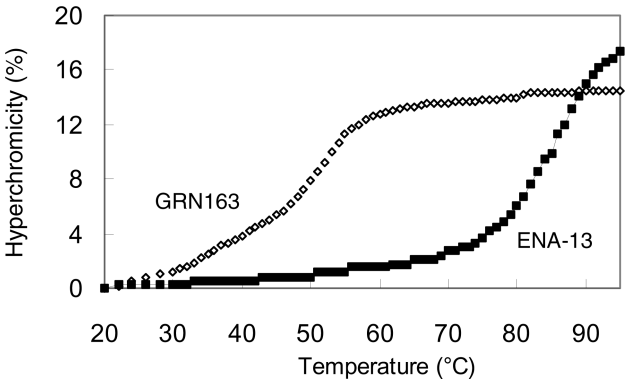


FIGURE 5 T_m values of duplexes of ENA-13 (closed squares) and GRN-163 (open diamonds) with complementary RNA, 5′-UUG UCU AAC CCU A-3′. Duplex concentration: 3.4 μM; buffer: 10 mM sodium phosphate buffer (pH 7.2).

ENA-13 and GRN163, 5′-fluorescein-labeled ENA-13, 5′-FAM-ENA-13, and 3′-fluorescein-labeled GRN163, 3′-FAM-GRN163, were synthesized and added to human prostate cancer DU-145 cell cultures. After 24 h of incubation at concentrations of 0.1, 1, and 10 μM of the oligonucleotides, fluorescence was measured using flow cytometry. Cellular uptake levels of 5′-FAM-ENA-13 and 3′-FAM-GRN163 at all the concentrations were almost identical as shown in Figures 2A and 2B (data shown for 1 μM only).

Oligonucleotides with NPS bonds are hydrolyzed under acidic conditions because of protonation of the nitrogen atom in the NPS.[35] Since it is known that the pH values of endosomes and lysosomes of cells are around pH 5.0–5.5, the stability of oligonucleotides under acidic conditions would be important for their delivery into cells via endosomes and lysosomes by endocytosis.[36,37] When we compared the stability between ENA-13 and GRN163 in a solution of 20 mM sodium acetate (pH 5.0) at 37°C, the remaining GRN163 was decreased by 40% after 24 h incubation (Figure 2C) and some peaks with shorter retention time than that of GRN 163 were observed in AE HPLC analysis (data not shown). On the other hand, no ENA-13 was changed even after 100 h incubation (Figure 2C). These results indicate that ENA-13 is much more stable than GRN 163 under acidic conditions and also suggest that GRN163 might be degraded and less effective than ENA-13 in acidic endosomes and lysosomes during cellular uptake, even though identical cellular uptake of ENA-13 and GRN163 is shown in Figures 2A and 2B.

Summary

ENA-13 and GRN163, an ENA PS oligonucleotide and NPS oligonucleotide, respectively, were compared in terms of synthesis and properties

for antisense application: (a) Although isolated yield of GRN163 was very low, ENA-13 could successfully be synthesized on a 100-mg scale. (b) ENA-13 and GRN163 did not form the G-quadruplex that is not suitable as an antisense molecule. (c) ENA-13 formed a rigid structure as a single strand. Once ENA-13 was incubated with the complementary RNA, ENA-13 showed stronger binding to complementary RNA than GRN163. (d) Both ENA-13 and GRN163 showed superior cellular uptake without uptake enhancers; however, although ENA-13 might be stable in acidic endosomes and lysosomes, GRN 163 might be hydrolyzed. These four findings suggest that ENA-13 has superior properties to GRN163 for antisense therapeutic application, and that ENA-13 is a promising candidate as an antisense molecule. We await biological testing of ENA-13 in comparison with GRN163.

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